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Mechanisms of entry of amastigotes of *Leishmania major* into macrophages.

by

Rebecca Ann Guy

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biological Sciences

Edmonton, Alberta

Fall 1995

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ABSTRACT

The interaction of macrophages with the amastigote stage (tissue stage) of the intracellular protozoan parasite *Leishmania major* was studied to elucidate the mechanisms involved in the entry of this stage of the parasite into its host cell. Two sources of amastigotes, that differed in the host components bound to their surface were compared: amastigotes recovered from BALB/c mice had host immunoglobulins and complement component C3 bound to their surface whereas those recovered from *scid* (severe combined immunodeficient) mice had no surface immunoglobulin and little C3. The uptake of the amastigotes by different macrophage populations differed from each other and both differed from the promastigote stage (insect stage) of *Leishmania*. Opsonization of *scid*-source amastigotes with parasite-specific IgG enhanced the entry of amastigotes into macrophages; however, blocking the Fc receptor (FcR) did not reduce the entry of amastigotes. Blocking the complement type 3 receptor (CR3) reduced the entry of amastigotes by 40%. Simultaneous blocking of the FcR and CR3 reduced the entry of amastigotes by 60 to 70%. The mannose fucose receptor (MFR) was not used for the entry of either promastigotes or amastigotes.

The surface of amastigotes was examined to identify parasite.ligands that bind macrophage receptors. Three major surface molecules of 13, 11 and 9-5 kDa were detected using radioiodination. Biotinylation revealed 2 major molecules, 11 kDa and 66 kDa. Immunoblotting, using different parasite-specific antibodies, revealed only a few differences in the antigenic structure of the two sources of amastigotes, whereas both amastigotes differed greatly from promastigotes. A small molecular weight protein isolated from amastigotes was responsible for a 29% decrease in the entry of amastigotes

into macrophages, suggesting that the molecule is directly involved in binding live amastigotes to macrophage receptor(s).

This study identified two receptors, the FcR and CR3, used for entry of the amastigote stage of *L. major*, and the data suggests that the amastigotes use alternate receptors to enter into macrophages. In addition, a novel amastigote molecule was isolated that is involved in the entry of amastigotes into macrophages.

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LIST OF ABBREVIATIONS

2-D-PAGE: 2-Dimensional-Polyacrylamide Gel Electrophoresis

AGE-R: Advanced Glycosylation Receptor

ATCC: American Type Culture Collection

BCG: Bacillus Calmette Guerin

BMDM: Bone Marrow-Derived Macrophages

BSA: Bovine Serum Albumin

ConA: Concanavalin A

CPM: Counts Per Minute

CR1: Complement Receptor type 1

CR2: " " " 2

CR3: " " " 3

CSF-1: Colony Stimulating Factor-1

DMEM: Dulbecco's Modified Eagles Medium

DMSO: Dimethyl Sulfoxide

DNP: Dinitrophenol

EB: Ethidium Bromide

ELISA: Enzyme-Linked Immunosorbent Assay

FBS: Fetal Bovine Serum

FcR: Fc Receptor

FDA: Fluorescene Diacetate

FITC: Fluorescein Isothiocyanate

FML: Fucose Mannose Ligand

FnR: Fibronectin Receptor

FPLC: Fast Protein Liquid Chromatography

GIPL: Glycoinositol Phospholipid

GM-CSF: Granulocyte Macrophage-Colony Stimulating Factor

gp63: Glycoprotein 63

GPS: Guinea Pig Serum

GSL: Glycosphingolipid

HRP: Horse Radish Peroxidase

ICAM: Intercellular Adhesion Molecule

IFA: Indirect Immunofluorescence Assay

IFNγ: Interferon gamma

Ig: Immunoglobulin

IL- Interleukin

iNOS: inducible Nitric Oxide Synthase

iPC: inflammatory Peritoneal Cells

kDa: kilodalton

KMP-11: Kinetoplastid Membrane Protein-11

LCCM: L-cell Conditioned Medium

LFA-1: Lymphocyte Activation Antigen

LPG: Lipophosphoglycan

LPS: Lipopolysaccharide

mAb: Monoclonal Antibody

MFL: Mannose Fucose Ligand

MFR: Mannose Fucose Receptor

MMR: Macrophage Mannose Receptor

MSP-1: Merozoite Surface Protein

MW: Molecular Weight

NGPS: Normal Guinea Pig Serum

NK cells: Natural killer cells

NO_2: Nitrite

NOC: Nocodazole

OAG: Oleoylacetylglycerol

O.D.: Optical Density

PBS: Phosphate Buffered Saline

PHA: Phytohemagglutinin

PI-PLC: Phosphoinositol-Phospholipase C

PMA: Phorbol Myristate

PNA: Peanut Agglutinin

PSA-2: Promastigote Surface Antigen-2

RCA: Ricinus Agglutinin

RGD: Arginine-Glycine-Aspartic acid

rPC: resident Peritoneal Cells

SBA: Soybean Agglutinin

***scid*:** severe combined immunodeficient

SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Th: T helper

TNF-α: Tumor necrosis factor-alpha

VLA-5: Very Late Antigen-5

WGA: Wheat Germ Agglutinin

Chapter I: Introduction and Literature Review.

INTRODUCTION

The genus *Leishmania* is a group of intracellular protozoans that are obligate parasites of macrophages of humans and other animals. The uncontrolled spread of the tissue stage amastigotes from macrophage to macrophage results in the disease, leishmaniasis, manifested as three basic clinical syndromes: cutaneous, mucocutaneous and visceral. Infection with *Leishmania major* results in the cutaneous form of the disease which is known as Oriental sore (Jericho boil, Allepo boil or Delhi boil). The infection is manifested by a localized lesion at the site of the sandfly bite. This lesion usually heals within several months, leaving a depressed, unpigmented scar, and life long immunity to reinfection. Shown in Table 1-1 are the different species of *Leishmania* that I will refer to in this thesis, the forms of leishmaniasis they cause, and their geographical distribution.

The life cycle of *Leishmania* involves two stages (Figure 1-1). The extracellular promastigote is the motile stage, adapted to living in the midgut of the sandfly. The promastigote is 10-15 μm in length with a diameter of 1-2 μm and possesses a single anterior flagellum. The tissue stage in the life cycle is the amastigote which resides in the phagolysosome of the macrophage. The amastigote is round with a diameter of 2-5 μm, it lacks the flagellum and consequently is non-motile. Infection with *Leishmania* is acquired through the bite of a sandfly. In the tissues, promastigotes rapidly enter into macrophages and transform into amastigotes. The amastigotes replicate within the macrophages, eventually destroying the host cell, and invade other macrophages present at the inflammatory lesion site.

The monocyte/macrophage are the only cells *Leishmania* survive and replicate in, implying a high degree of specificity in the recognition of these cells by the parasite. Entry into macrophages involves recognition of specific parasite ligands by receptors on the macrophage surface, and eventual internalization of the parasite by the macrophage. Much is known about the entry of the promastigote stage into macrophages, while virtually nothing is known about the entry of the amastigote into the host cell. The focus of my thesis research was to elucidate the mechanisms of entry of the tissue stage, the amastigote, into macrophages.

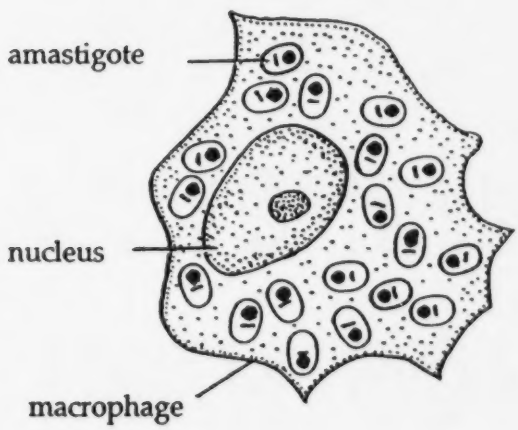
Table 1-1: The geographical distribution and disease forms of *Leishmania* species.

DISEASE FORM	SPECIES	DISTRIBUTION
Cutaneous	*L. tropica*	Middle East, South Europe
	L. tropica major (= *L. major*)	Middle East, Africa, South Europe
	L. mexicana mexicana	Central America
	L. m. amazonensis	Mexico-Brazil
Mucocutaneous	*L. braziliensis braziliensis*	Latin America
	L.b. guyensis	Guyana
	L.b. panamensis	Panama
Visceral	*L. donovani*	Asia, Africa
	L. d. chagasi (= *L. chagasi*)	Central and South America

SANDFLY

Replication of promastigotes
in the mid gut of the sandfly

MAMMALS



Replication of amastigotes in
the macrophage

Figure 1-1: Life cycle of *Leishmania*.

In the first chapter I review the literature pertaining to entry of *Leishmania* into macrophages. The second chapter provides a detailed description of the general materials and methods that were employed. Chapter III contains the results of studies on the characterization of the *scid* mouse model used as the primary source of amastigotes in this study. In Chapter IV, I describe the basic parameters of entry of the parasite into macrophages in vitro. Chapter V contains data on the macrophage receptors utilized by amastigotes to gain entry into their host cell. In Chapter VI, I characterize the amastigote surface, identifying molecules that potentially bind to macrophages. The data in chapter VII are preliminary findings on the blocking of entry of amastigotes into macrophages in vitro, by parasite ligands. The final Chapter, VIII, is a general discussion, wherein I summarize the findings and discuss future directions of research in this area.

LITERATURE REVIEW [1]

This review is a synthesis of information from the literature regarding the surface molecules of the two stages of *Leishmania*, their involvement in entry of the parasite into the host cell and the macrophage receptors involved in uptake of the different *Leishmania* spp. Since the majority of the research in this area has been done with the promastigote stage, the bulk of this review contains information regarding the promastigote; however, an understanding of the mechanisms involved in entry of the promastigote stage provides a basis for studying the amastigote stage. Data on amastigotes is included whenever available.

Parasite Surface:

It has been established that the two stages of *Leishmania*, the promastigote and amastigote, share certain surface molecules, whereas others are unique to each stage. For example, Dwyer (1974) demonstrated that when anti-amastigote serum is pre-absorbed with promastigotes, the intensity of fluorescence is reduced compared to non-adsorbed serum, indicating the loss of common antigens. However, the amastigotes still fluoresce, indicating the presence of unique or amastigote-specific molecules. Macrophages infected

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[1] This review is an updated version of one we published: Guy and Belosevic. Adv. Struct. Biol. 1991. 1:301-24.

with *L. donovani* possess amastigote-specific antigens on their surface that are not recognized by sera to the promastigote stage (Berman and Dwyer, 1981). Sadick and Raff (1985a) reported that the proliferative response of T cells, obtained from lymph nodes of immunized mice, was greater to promastigote antigens than to amastigote antigens, further demonstrating the differences in antigenic structure of the two stages. A number of studies have also identified major surface antigens common to all *Leishmania* (Colomer-Gould *et al.*, 1985; Etges *et al.*, 1985; Tolson *et al.*, 1994b); however, species specific antigens exist (Jaffe and McMahon-Pratt, 1983; Jaffe and Sarfstein, 1987; Kahl and McMahon-Pratt, 1987).

Using a variety of techniques, such as surface labeling and SDS-PAGE, immunoprecipitation and immunoblotting several surface antigens have been described for the promastigote stage of *Leishmania*. Fewer studies have been conducted to identify amastigote surface antigens, and several studies compared antigens in both stages of the parasite. The results of these studies are listed in Table 1-2.

The surface of *Leishmania* also contains glucose/mannose-like residues, identified by lectin binding. Binding of lectins is distributed randomly on the entire surface of the organisms and flagella, using cytochemical visualization and transmission electron microscopy (Dwyer, 1977). Differences in the degree of lectin binding are evident within species, between species and also between developmental stages of *Leishmania*. Dwyer (1974) showed that *L. donovani* promastigotes were agglutinated by concanavalin A (ConA) and phytohemagglutinin-P (PHA-P) but not PHA-M. Promastigotes also bind to soybean agglutinin (SBA), wheat germ agglutinin (WGA) and influenza virus (Dwyer, 1977). In contrast, Jacobson *et al.* (1982) did not find that PHA-P agglutinated the strains they studied, including *L. donovani*; different strains reacted to either ConA, SBA or ricinus agglutinin (RCA). Gardiner and Dwyer (1983) reported that Con A precipitated 10 surface iodinated proteins of *L. tropica*, which are recognized by a rabbit antiserum against the pellicular membrane. Other lectins did not precipitate iodinated surface components.

Differences in lectin binding between promastigotes and amastigotes also exist. Both stages bind Con A and RCA; ConA binding to promastigotes is inhibited by D-mannose and to amastigotes by α-methyl-mannopyranose.

Table 1-2: Surface Antigens of *Leishmania*.

Species	Molecular Weight (kDa)	Established by	Reference
		AMASTIGOTES	
L. major	94, 43	Radioiodination & 2D Immunoprecipitation	Handman and Curtis, 1982
L. major	250, 165, 90, 66, 35, 31	Radioiodination & Immunoprecipitation	Handman *et al.*, 1984b
L. major	57, 47, 35, 24, (weak) 9.5 (strong)	Radioiodination & SDS-PAGE	Sadick and Raff, 1985b
L. major	180, 14 doublet	Immunoprecipitation using mAbs	Jaffe and Rachamin, 1989
L. braziliensis	260, 130, 85-75, 12	Immunoblot using mAbs	Eperon and McMahon-Pratt, 1989
L. mexicana	65, 52-48	Radioiodination & Immunoprecipitation	Hernandez *et al.*, 1989

Species	Molecular Weight (kDa)	Established by	Reference
		PROMASTIGOTES AND AMASTIGOTES	
L. major	80, 70, 60, 50	Radioiodination & 2D Immunoprecipitation	Handman and Curtis, 1982
L. major	15-10	Immunoblot using mAbs	Elhay *et al.*, 1988
L. major & *L. mexicana*	63	IFA & Immunoblot	Frommel *et al.*, 1990
L. mexicana mexicana	63	Immunoprecipitation Peptide mapping	Medina Acosta *et al.*, 1989
L. mex. mexicana *L. brazil. guyensis,* & *L. brazil. brazil.*	65	Radioiodination & Immunoprecipitation	Colomer-Gould *et al.*, 1985
L. panamensis	25, 20, 16	Immunoblot	Eperon and McMahon-Pratt, 1989
L. brazil. panamensis	15	Immunoblot using mAbs	Williams *et al.*, 1986a

Species	Molecular Weight (kDa)	Established by	Reference
		PROMASTIGOTES	
L. major	260-10	Radioiodination & SDS-PAGE	Gardiner and Dwyer, 1983
L. major	71, 64, 58,53, 48, 43, 9.5	Radioiodination & SDS-PAGE	Sadick and Raff, 1985a
L. major	116-62	Immunoprecipitation & Immunoblot	Sacks *et al.* 1987
L. major	200, 100, 70, 50	Radioiodination & Immunoprecipitation using mAbs	Jaffe and McMahon-Pratt, 1983
L. major	116, 80, 58, 50	Radioiodination & Immunoprecipitation	Sacks *et al.*, 1985
L. major	96, 80, 50	Immunoblot using mAbs	Symons *et al.*, 1994
L. major	66, 50	Radioiodination & 2D Immunoprecipitation	Handman and Curtis, 1982
L. major	67-20	Immunoprecipitation using mAbs	Handman *et al.*, 1984b

Species	Molecular Weight (kDa)	Established by	Reference
		PROMASTIGOTES	
L. tropica	92, 80, 62, 55	Radioiodination & Immunoprecipitation using mAbs	Jaffe and Sarfstein, 1987
L. tropica	46-26	Immunoprecipitation using mAbs	Jaffe *et al.*, 1990
L. donovani	93.8, 97.6, 65.7, 53, 18	Radioiodination & SDS-PAGE	Ramasamy *et al.*, 1981
L. donovani	65, 25-23	Radioiodination & SDS-PAGE	Lepay *et al.*, 1983
L. donovani	115, 84, 74, 60, 18.5, 20.5	Radioiodination & Immunoprecipitation using mAbs	Jaffe *et al.*, 1984
L. donovani	86, 66, 53	Immunoblot	Singla and Vinayak, 1994
L. donovani	60, 40, 35	Immunoblot using mAbs	Symons *et al.*, 1994
L. mexicana	65, 48, 27 94, 65, 48-52, 27	Radioiodination Immunoprecipitation	Hernandez *et al.*, 1989

Species	Molecular Weight (kDa)	Established by	Reference
		PROMASTIGOTES	
L. mexicana	46	Immunoblot using mAbs	Symons *et al.*, 1994
L. mexicana amazonensis	90, 68 (triplet)	Immunoprecipitation using mAbs	Fong and Chang, 1982
L. mexicana amazonensis	66-60 (4 bands)	Immunoaffinity column using mAbs	Chang *et al.*, 1986
L. braziliensis	65, 54-47, 25	Radioiodination & SDS-PAGE	Misle *et al.*, 1985
L. braziliensis	65, 50	Radioiodination & Immunoprecipitation using mAbs	Kweider *et al.*, 1987
L. braziliensis *L. amazonensis,* & *L. major*	13.5	Immunoblot using -mAbs	Ismach *et al.*, 1989

RCA binding is inhibited by lactose for both stages. During the transformation from amastigotes to promastigotes, changes in the degree of lectin binding have been observed. For example, amastigotes bind WGA, but by the second day of transformation WGA binding is lost and the transformed promastigotes bound peanut agglutinin (PNA) instead. In early promastigotes, PNA was located primarily in the flagellar pocket and as promastigotes matured, bound PNA was evident on the entire surface of the parasites (Wilson and Pearson, 1984).

In addition to containing surface sugar residues, promastigotes also possess a net negative surface charge seen by agglutination and electron microscopy, using cationized ferritin (Dwyer, 1977; Saraiva *et al.*, 1989). The biological significance of a net negative charge on the parasite surface remains to be determined, but it may be involved in *Leishmania*-macrophage binding (Saraiva *et al.*, 1989). A negative charge on the parasite surface may also prevent nonspecific binding of *Leishmania* to cells, due to electrostatic repulsion.

From these studies a number of major surface antigens have been identified and I will discuss each one separately, reporting on their physicochemical characteristics and biological functions within the cell.

Glycoprotein 63 (gp63):

A 63 to 65 kDa protein was the first surface glycoprotein to be characterized in *Leishmania*. It is known as glycoprotein 63 (gp63) or the promastigote surface protease (PSP). A 63 or 68 kDa band is seen in promastigotes of all species of *Leishmania* when the promastigotes are surface labeled and analyzed by SDS-PAGE (Gardiner and Dwyer, 1983; Colomer and Gould, 1985; Etges *et al.*, 1985). When *L. donovani* and *L. major* promastigotes are radiolabeled with ^{125}I, 70% of the label was incorporated into gp63 (Etges *et al.*, 1985). Gp63 was also observed in immunoblots using mAbs (Fong and Chang, 1982) and sera from patients infected with *L. donovani* or *L. major* (Etges *et al.*, 1985; Heath *et al.*, 1987). A 65 kDa molecule was recognized by the sera of patients with various clinical forms of leishmaniasis, in immunoprecipitation using both promastigotes and amastigotes as antigen for the assay (Colomer-Gould *et al.*, 1985). An ELISA, using *L. donovani* gp63 as the antigen, was used to detect the presence of anti-gp63 antibodies in the serum of patients with visceral leishmaniasis. The test was positive for 97.3%

of the confirmed cases of leishmaniasis (Vinayak *et al.*, 1994). Gp63 was also recognized by the serum of a dog naturally infected with *L. donovani* (Etges *et al.*, 1985).

Although all species of *Leishmania* possess this major surface antigen there is a slight variation in its molecular weight (MW), ranging from 63 to 68 kDa (Fong and Chang, 1982; Hernández *et al.*, 1989; Colomer-Gould, *et al.*, 1985). The basic peptide core is the same in different species; differences occur in the level of glycosylation (Colomer-Gould, *et al.*, 1985). The conserved structure of this molecule indicates a biological function necessary for survival of all *Leishmania* species (Etges *et al.*, 1985).

Gp63 comprises up to 1% of the total protein of promastigotes of *L. major* (Etges *et al.*, 1985; Bouvier *et al.*, 1985) and is distributed on the entire surface and flagellum (Russell and Wilhelm, 1986), with a surface density of approximately $5x10^5$ molecules per cell (Bouvier *et al.*, 1985). Although it is relatively abundant, it does not form a coat of closely packed molecules as is the case with the variable surface glycoproteins of African trypanosomes (Bordier, 1987). It is found in higher levels on infective than non-infective promastigotes (Kweider *et al.*, 1987). Using immunoelectron microscopy, Pimenta *et al.*, (1991) demonstrated very little labeling of gp63 on the surface of *L. major* promastigotes. Soluble forms of gp63 also exist, either due to hydrolytic cleavage from the membrane bound form, as in *L. major* (Bouvier *et al.*, 1985) or released from cytoplasmic stores in *L. (b.) braziliensis* and *L. (d.) chagasi* (Kutner *et al.*, 1990).

The gene encoding the gp63 molecule of promastigotes of *L. major* has been cloned (Button *et al.*, 1988). Gp63 is synthesized as a precursor molecule and the mature form, expressed on the surface, is a protein of 601 amino acids. This molecule is encoded by a small multi-gene family with the genes arranged as 5 tandem repeats at a single chromosomal locus (Button *et al.*, 1989). Other studies have suggested that gp63 is located on 2 chromosomes (Hanekamp and Langer, 1991; Maignon *et al.*, 1990). The primary structure and organization of this gene is highly conserved among most species of *Leishmania* (Button *et al.*, 1988). Homology of 88-92% between *L. major* and *L. chagasi* and *L. donovani*, was reported (Miller *et al., 1990;* Webb *et al.*, 1991). The gp63 gene for *L. guyensis* is more divergent with approximately 70% homology to the other species of *Leishmania* (Steinkraus and Langer, 1992). Conserved in all the species examined are the putative functional sites for

membrane anchor attachment, the zinc-binding domains and 2 of 3 potential glycosylation sites (Webb *et al.*, 1991; Steinkraus and Langer, 1992). The first report of the DNA sequence of *L. major* identified an RGD (arginine-glycine-aspartic acid) sequence at position 375 (but no serine as in the RGDS sequence), suggesting the presence of a region of homology between gp63 and fibronectin (Button *et al.*, 1988). It was postulated that gp63 may be fibronectin-like (Rizvi *et al.*, 1988). However, Bouvier *et al.* (1989) examined the structure of gp63 using circular dichroism spectroscopy and reported that the molecule contains mainly β-pleated sheets and a relatively modest content of α-helixes, suggesting that gp63 is "anything but fibronectin-like". Further examination of the sequence of gp63 has revealed that there is no RGD sequence in either *L. major* or *L. chagasi* (Button and McMaster, 1990; Miller *et al.*, 1990).

Antibody-induced capping of gp63 occurs in promastigotes, indicating that gp63 is mobile on the membrane surface (Chang *et al.*, 1986; Fong and Chang, 1982). The anchor of gp63 is a glycosyl-inositolphospholipid (GPI) (Bordier *et al.*, 1986; Schneider *et al.*, 1990). The anchor consists of a COOH-terminal tetrapeptide of the protein linked to a carbohydrate core by an ethanolamine phosphate bridge and the inositol phospholipid contains an α-1-O-alkyl-2-O-acylglycerol moiety (Schneider *et al.*, 1990).

Gp63 contains carbohydrate moieties since it binds concanavalin A (ConA) and lentil agglutinin (Lepay, 1983; Bouvier *et al.*, 1985), wheat germ agglutinin (WGA) and *Helix pomatia* lectin (Russell and Wilheim, 1986). The carbohydrate content of gp63 is estimated at 10% (Bouvier *et al., 1985*) and consists mainly of *N*-linked oligosaccharides (Chang *et al.*, 1986; Olafson *et al.*, 1990). Treatment of promastigotes with tunicamycin, a specific inhibitor of N-acetylglucosaminyltransferase, alters the elecrophoretic mobility of gp63, shifting from a 63 to a 50-54 kDa band (Chang *et al.*, 1986; Wilson and Hardin, 1990; Funk *et al.*, 1994). Some functions for the carbohydrates have been postulated. Wilson and Hardin (1990) reported that the deglycosylated form of gp63 produced in tunicamycin resistant promastigotes is proteolytically inactive, whereas Funk *et al.* (1994) demonstrated that tunicamycin treatment of normal promastigotes has no effect on protease activity or the ability of gp63 to resist proteolysis by cathepsin B.

Gp63 appears to be a major antigen of the tissue form of *Leishmania*, the amastigote, although there is controversy over expression of gp63 in this stage. The findings of surface labeling studies of amastigotes, using

iodination, have yielded contradictory results. A major 63-65 kDa band is observed in different species of *Leishmania* (Handman *et al.*, 1984b; Colomer-Gould *et al., 1985*), whereas other researchers were unable to detect a major 63 kDa band in iodinated amastigotes (Sadick and Raff 1985b; Schneider *et al.*, 1992). However, a major 60 kDa band in *L. mexicana* and 66 kDa band in *L. major* is visualized when the amastigotes were surface labeled using biotin (Medina-Acosta *et al.*, 1989). Studies using SDS-PAGE analysis of *L. mexicana* indicate that both promastigotes and amastigotes possess gp63, and the peptide backbone appears to be identical for the two stages of the parasite (Medina-Acousta *et al.*, 1989). There are differences in the migration of gp63 from metabolically labeled cells run on SDS-PAGE; the amastigote antigen runs as a complex of 60-72 kDa. There are some epitopes within gp63 that are stage specific, for example, anti-gp63 mAbs against promastigotes of *L. mexicana amazonensis* did not bind to the surface of amastigotes in IFA, nor to blots of amastigote antigens (Chang *et al.*, 1986). In contrast, polyclonal rabbit serum against *L. mexicana* or *L. major* promastigote gp63, and anti-recombinant gp63 serum, recognize amastigotes in immunoblots (Chaudhuri *et al.*, 1989; Frommel *et al.*, 1990). On the other hand, Schneider *et al.* (1992) did not observe bands when amastigote antigen was blotted against a rabbit anti-promastigote gp63 serum. Interestingly, Medina-Acousta *et al.*, (1989) reported that there are several forms of gp63 expressed in the amastigote, seen using immunoprecipitation of [^{35}S]-labeled amastigotes in 2-dimensional polyacrylamide electrophoresis (2-D-PAGE), but, only a few of these forms are expressed on the surface, as only a few bands are seen in [^{3}H]myristic acid-labeled amastigotes analyzed by 2-D-PAGE. The surface form of gp63 has the phosphatidylinositol tail as evidenced by [^{3}H]myristic acid-labeling and release of gp63 after phosphoinositol-phospholipase C (PI-PLC) treatment (Medina-Acousta, *et al.*, 1989). No gp63 was detected in the detergent extracts of iodinated or metabolically labeled amastigotes (Heath *et al.*, 1987; Schneider *et al.*, 1992), while Chaudhuri *et al.* (1989) found the 63 kDa band when iodinated amastigotes were immunoprecipitated using rabbit anti-promastigote serum.

Some researchers have postulated that gp63 is constitutively expressed in both stages of the life cycle. Button *et al.* (1989) detected gp63 gene transcripts in both stages, and Heath *et al.* (1987) obtained similar translation profiles of gp63 from mRNA of promastigotes and amastigotes, whereas

Schneider *et al.* (1992) reported that the mRNA for gp63 is down regulated in the amastigote stage. A major difference between amastigotes and promastigotes is where gp63 is located. Metabolic and surface labeling revealed that most of gp63 is on the surface of promastigotes; in contrast, small amounts of unevenly distributed gp63 appears on the surface of amastigotes and are concentrated mainly in the flagellar pocket (Medina-Acousta *et al.*, 1989) or in the lumen of the megasomes in *L. mexicana* (Bahr *et al.*, 1993). Pimenta *et al.* (1991) found using immunoelectron microscopy that anti-gp63-coated gold particles bound to the amastigote surface in greater amounts than to the promastigote surface. The distribution of gp63 is linear on the amastigote surface, with some patches. Certain mAbs as well as polyclonal antibodies raised against purified gp63, and recombinant gp63 recognize *L. mexicana* and *L. major* amastigotes inside macrophages by IFA (Medina-Acousta *et al.*, 1989; Frommel *et al.*, 1990).

The discrepancy between results of studies designed to determine the presence and location of gp63 in the amastigote stage is difficult to explain. Isolate variation may account for the contradictory results using the same species, since different isolates from the same species of *Leishmania* express different amounts of gp63 on the promastigote surface (Bouvier *et al.*, 1987). In addition, it seems apparent that gp63 is expressed in amastigotes but at lower levels than the promastigote, therefore, a low level of activity may have caused the negative results. A bias towards negative results is possible if equivalent numbers of amastigotes and promastigotes are used in the assays, since the amastigote is much smaller and contains over 5 times less protein than an equivalent number of promastigotes.

A number of functions of gp63 in the biology of *Leishmania* have been postulated. One of the roles is in binding macrophage receptors for entry into the host cell. This role will be covered in detail in the next section of the review. Gp63 is also involved in the survival of *Leishmania* within macrophages. Tunicamycin resistant promastigotes, lacking the carbohydrate moieties on gp63, infect, but do not survive within macrophages in vitro, and are non-infective to BALB/c mice (Wilson and Hardin, 1990). The level of gp63 expression; however, does not correlate with increased survival, since an avirulent strain of *L. major* expressed more gp63 than the virulent clones (Murray *et al.*, 1990). Interestingly, a sinefungin-resistant clone of *L. donovani*

that expresses more gp63 than the wild type parasite, was equally virulent in mice (Soteriadou *et al.*, 1994).

Another important function of gp63 is as a protease, hence its name the promastigote surface protease (PSP) (Etges *et al.*, 1986). All *Leishmania* spp. possess gp63 protease activity; however, levels of activity vary depending on the species and strain of *Leishmania* and this correlates with the level of gp63 expressed on the cell surface (Bouvier *et al.*, 1987; Tzinia and Soteriadou, 1991). There are two forms of gp63: an amphiphilic form with a phospholipid membrane anchor and a hydrophilic form, a product of cleavage of the anchor by lipase activity. The proteolytic activity against azocasein, by these two forms is of equal specific activity and is 1.5 times greater than trypsin. The protease is not excreted and proteolytic activity requires promastigote-cell contact (Etges *et al.*, 1986). Several lines of evidence demonstrate that the gp63 is a metalloprotease: 1) proteolytic activity is inhibitable with metal chelating agents (Etges *et al.*, 1986; Chaudhuri *et al.*, 1989); 2) there is one atom of zinc per gp63 molecule, as seen by emission spectroscopy and the incorporation of $^{65}ZnCl_2$; 3) the amino acid sequence for gp63 contains the zinc-binding site, [-Glu-His-X-X-His-], identical to 9 other metalloproteases; 4) the sequence for gp63 also contains a glutamyl residue thought to be responsible for hydrolysis of the substrate (Bouvier *et al.*, 1989). A mutant promastigote created by site directed mutagenesis by changing the glutamic acid to an aspartic acid, produced a form of gp63 that lacked proteolytic activity (McMaster *et al.*, 1994). The hydrophilic form of gp63 is inhibited by the protease inhibitor α-2 macroglobulin, whereas the amphiphilic form is not affected by this protease inhibitor because complexes of α-2 macroglobulin do not form on the promastigote surface (Heuman *et al.*, 1989). The cleavage site for *L. m. mexicana* gp63 is at the amino side of a serine or threonine residue (Ip *et al.*, 1990).

Controversy over the optimum pH range for PSP proteolytic activity exists; this is not surprising since the studies were performed with different species of *Leishmania* and different substrates. *L. m. mexicana* gp63 is active at acidic pH against bovine serum albumin (BSA), with an optimum activity at pH 3-3.5 (Chaudhuri and Chang, 1988; Chaudhuri *et al.*, 1989). *L. major* gp63 displays proteolytic activity against azocasein at pH 5-11 with peak activity at pH 7-10 (Etges *et al.*, 1986). Proteolytic activity of *L. mexicana* gp63, using peptides as a substrate, was found to optimal at pH 7.5 (Ip *et al.*, 1990). Tzinia

and Soteriadou (1991) demonstrated that the optimum pH is substrate dependent. Optimum activity against azocasein occurred at neutral to alkaline pH, whereas the optimal activity against the β-chain of insulin was at pH 6-6.5. The same results were obtained when whole promastigotes were compared to purified gp63 from 7 strains of *Leishmania* (Tzinia and Soteriadou, 1991).

Amastigote gp63 also has protease activity (Chaudhuri *et al.*, 1989), detected by immunoprecipitation of a detergent solubilized extract using a rabbit anti-promastigote gp63 serum. The optimum proteolytic activity is at pH 3-3.5 with a range from pH 2.5-5.5 (Chaudhuri *et al.*, 1989). A soluble form of gp63 isolated from *L. mexicana* amastigotes has optimal activity against azocasein at pH 5.5-6 (Ilg *et al.*, 1993). Only a tenth of the amount of gp63 in promastigotes was present in amastigotes (Ilg *et al.*, 1993). The importance of amastigote gp63 as a protease in the biology within the host is questionable due to the low levels of gp63 expressed in amastigotes. However, three distinct groups of 20-35 kDa cysteine proteases have been identified in *L. m. mexicana* amastigotes (Robertson and Coombs, 1990); these low molecular weight proteases are localized within the megasomes (Ilg *et al.*, 1994). Two genes (Lpcys1 and Lpcys2) have been identified for the *L. mexicana* cysteine proteinases. Related genes also exist in *L. major*, *L. donovani* and *L. braziliensis*, although in lower copies than in *L. mexicana* (Traub-Cseko *et al.*, 1993).

A number of functions for the proteolytic activity of gp63 have been hypothesized, such as entry into the host cell, and enhancement of lesion development (Ilg *et al.*, 1994; McMaster *et al.*, 1994). Gp63 protease activity may protect the parasite from proteolytic degradation within the macrophage, as liposomes coated with gp63 are not degraded within macrophages (Chaudhuri *et al.*, 1989). One potential natural substrate for gp63 is the CD4 molecule on T cells. Incubation of T cells with gp63 results in a reduction in binding of mAbs to CD4 (Hey *et al.*, 1994). Proteolytic cleavage of CD4 by gp63 could enhance parasite infectivity by interfering with the activation of T cells.

Gp63 may also play a role as a virulence factor in the extracellular environment at the lesion site. Sørensen *et al.* (1994) demonstrated that gp63 inhibits the chemotaxis of neutrophils in vitro, in response to f-met-leu-phe and inhibited, to a lesser degree, the chemotactic response of macrophages to zymosan-activated serum containing C5a. Since neutrophils are cytotoxic to

Leishmania, inhibiting their chemotaxis may help in preventing destruction of the parasite. In addition, gp63 reduced the chemiluminescence response of macrophages and neutrophils to opsonized zymosan, suggesting that gp63 may reduce the level of H_2O_2 produced by macrophages, thereby preventing parasite death (Sørensen *et al.*, 1994).

Gp63 is a vaccination candidate and a number of studies have been conducted to determine its suitability as a vaccine. Semi-purified promastigote extracts containing an abundance of gp63 protect mice against primary and challenge cutaneous leishmaniasis (Murray *et al.*, 1989a; Frommel *et al.*, 1988). Promastigote gp63 incorporated into liposomes gives some protection in resistant CBA/Ca mice infected with *L. m. mexicana;* however, little protection was seen in susceptible BALB/c mice. A combination of gp63 and lipophosphoglycan (LPG) incorporated into liposomes gave better protection in the BALB/c mice, but protection was not complete, only 3/5 mice were uninfected (Russell and Alexander, 1988). Recombinant gp63 expressed as a fusion protein did not provide protection against *L. major* in either genetically susceptible or resistant mice. In addition, susceptible BALB/c mice did not recognize this antigen as they produced very little T-dependent antibodies to recombinant gp63, whereas resistant strains of mice produced a strong anti-gp63 antibody response (Handman *et al.*, 1990). Responses to gp63 are genetically controlled by both H-2 and non-H-2 linked genes; however, responses to gp63 do not correlate with resistance to *L. mexicana* (Lopez *et al.*, 1991).

Native or recombinant gp63 or defined gp63-peptides stimulate a strong proliferative response in T cells from *Leishmania* infected patients. Stimulation of cells results in IFNγ production but no IL-4 from patients with cured cutaneous and visceral leishmaniasis of New World species (Russo, *et al.*, 1991; Button *et al.* 1991) as well as patients with Old World species of cutaneous leishmaniasis (Kemp *et al.*, 1994). Therefore, gp63 induces a strong T helper 1 (Th 1) like response in humans.

A number of T-cell epitopes have been identified in gp63, in mice (Jardim, *et. al.*, 1990; Yang *et al.*, 1993; Soares *et al.*, 1994), vervet monkeys (Curray *et al.*, 1994) and in humans (Russo, *et al.*, 1991; Button *et al*, 1991). Examination of gp63 epitopes that induce T-cell responses revealed that a synthetic peptide corresponding to the conserved sequence involved in zinc binding, induces proliferation of a Th1 subset of $CD4^+$ T-cells. This peptide

was protective in susceptible BALB/c mice against infection with *L. major*, when inoculated s.c. in the presence of adjuvant. Interestingly the peptide alone exacerbated infection (Jardim, *et. al.*, 1990). A minimum length of 7 amino acids in the conserved sequence region are required for immunogenicity and include the 2 zinc-binding residues (Yang *et al.*, 1993).

Gp63 is also expressed on the surface of promastigotes within the sandfly gut and may play a role in the development of the parasite in the insect vector (Etges *et al.*, 1985; Grimm *et al.*, 1987).

Acid Phosphatases (AP):

Two forms of acid phosphatases have been identified from *Leishmania* promastigotes: a soluble form of 100-130 kDa that is secreted from the cell (Lovelace and Gottlieb, 1986; Bates and Dwyer, 1987; Menz *et al.*, 1991), and a surface bound, membrane associated form of *L. donovani* (Gottlieb and Dwyer, 1981; Glew *et al.*, 1982) and *L. mexicana*, that has an apparent molecular weight of 70 and 72 kDa and is also expressed in amastigotes (Menz *et al.*, 1991). Phosphatase activity of *L. major* is only 10% of the activity of *L. donovani* and *L. mexicana* (Menz *et al.*, 1991).

The substrates for the acid phosphatases as well as their function in the cell is unknown. A proposed role is in protection from the host cell. A membrane isolated AP from *L. donovani* promastigotes blocks the superoxide anion production by human neutrophils in vitro (Remaley *et al.*, 1984).

Two antigens of *L. donovani* have been identified that are immunogenic in humans and may serve a role in protection against the parasite. Legrand *et al.* (1987) reported a 72 kDa major surface antigen in isolates of *L. braziliensis* that was recognized by cutaneous and mucocutaneous human sera but not by Kala-azar or chagasic sera. Two surface antigens of 70 and 72 kDa isolated from *L. donovani* are recognized by serum from patients with visceral leishmaniasis and were recognized only by human visceral leishmaniasis sera (Jaffe and Zalis, 1988). Administration of these two antigens to mice prior to inoculation of *L. donovani* revealed that a 72 kDa molecule was protective; an 85% reduction in the amastigote burden in liver biopsies was obtained (Jaffe *et al.*, 1990). Whether the 70 and 72 kDa antigens are acid phosphatases has not been determined.

Fucose Mannose Ligand (FML):

Another glycoprotein on the surface of promastigotes of *L. donovani* is the FML that contains 10% fucose and 47% mannose residues (Palatnik *et al.*, 1989). It is a 36 kDa antigen located on the surface of the cells, as detected by agglutination and IFA using rabbit anti-FML serum (Palatnik-de-Sousa *et al.*, 1993).

Purified FML blocks the entry of promastigotes and amastigotes into macrophages in vitro (Palatnik *et al.*, 1989; Palatnik-de-Sousa *et al.*, 1993). Inhibition of promastigote entry also occurs with FML from *L. m. amazonensis*, but blocking is species-specific to a degree, and this inhibition has been used as a tool to identify relationships between species (Palatnik *et al.*, 1990). Administration of FML in vivo, prior to infection with *L. donovani*, resulted in 84% reduction of parasite burden in the livers of mice (Palatnik-de-Sousa *et al.*, 1994). Therefore, the FML has a role in entry of the parasite into macrophages and may also be a vaccine candidate. The importance of the FML in entry of other *Leishmania* species is not known.

Gp46/M-2:

A 46 kDa glycoprotein on the surface of *L. mexicana* was first identified using species- and stage-specific mAbs (Williams *et al.*, 1986a). It represents 1% of promastigote membrane proteins, contains 5 disulphide bonds and a low average hydrophobicity and it is very resistant to proteolytic degradation (Kahl and McMahon-Pratt, 1987). It is a glycoprotein with a 3 kDa *N*-linked carbohydrate side chain and is anchored to the membrane by a glycoinositol phospholipid (GIPL) anchor (Rivas *et al.*, 1991). The secondary structure of the glycoprotein consists primarily of turn structures and random coils with a minor portion of α-helical structure (Rivas *et al.*, 1991). Gp46 is expressed in *L. mexicana*, *L. major* and *L. donovani*, but not in *L. braziliensis* (McMahon-Pratt *et al.*, 1992).

Purified gp46 partially protects mice when administered in the presence of certain adjuvants. A delay in lesion development and lower numbers of parasite at the lesion site were observed in 2 susceptible strains of mice, the BALB/c and the CBA, when challenged with live promastigotes (Champsi and McMahon-Pratt, 1988). Interestingly, the glycoprotein exacerbated disease in mice that received gp46 alone and were challenged with a high dose of parasite.

It is also present on the surface of promastigotes within the midgut of the sand fly (McMahon Pratt *et al.*, 1983).

Promastigote Surface Antigen (PSA-2):

A complex of surface exposed glycoproteins of 94, 90 and 80 kDa are present in promastigotes of *L. major* (Murray *et al.*, 1989b). These antigens are anchored by a GIPL anchor on the parasite membrane, and are recognized by sera from patients with leishmaniasis (Murray *et al.*, 1989b). Water soluble forms of PSA-2 proteins are shed into the medium (Symons *et al.*, 1994). The PSA-2 proteins are encoded by a multigene family located on a single chromosome of *L. major*, *L. donovani* and *L. mexicana*. Several genes appear linked as tandem repeat arrays, while others are dispersed (Murray and Spithill, 1991). Homology between the PSA-2 genes occurs especially at their carboxy terminus.

There is a distinct pattern of expression of PSA-2 proteins of different species and isolates of *Leishmania*, as visualized using rabbit anti-PSA-2 antibodies and mAbs (Symons *et al.*, 1994). In *L. major*, bands of 96, 80 and 50 kDa were identified; however, different *L. major* isolates showed different bands. A weak 46 kDa band is obtained in immunoblots using *L. mexicana*, while using *L. donovani* bands of 60, 40 and 35 kDa were detected. In addition, a number of isoforms of the different bands are identified using 2-D blots (Symons *et al.*, 1994). Interestingly, when a genomic clone for an *L. major* gene was transfected into *L. mexicana* a 35 kDa molecule was expressed (Symons *et al.*, 1994). The function of proteins expressed by this highly polymorphic gene family in *Leishmania* is unknown.

Kinetoplastid membrane protein-11 (KMP-11):

The presence of a small glycoprotein on the surface of *Leishmania* promastigotes has been known for several years. It was called the lipophosphoglycan-associated protein because it is tightly associated with the glycan core of the glycolipid lipophosphoglycan (LPG) (Jardim *et al.*, 1991). Recently, the 11 kDa membrane protein has been identified and characterized. It has been renamed, KMP-11, since it occurs in all species of *Leishmania* as well in other trypanosomatids (Tolson *et al.*, 1994b). It is presumably a major surface protein of *Leishmania* promastigotes as it occurs at 1-2 x 10^6 copies per cell in *L. donovani*. KMP-11 has been detected in *L. donovani* amastigotes, by

reacting amastigote extracts with anti-KMP-11 mAbs using an ELISA; however, the location and expression levels in amastigotes is not known (Jardim *et al.*, 1995a). KMP-11 migrates on gels as a complex of 11-40 kDa bands and occurs either as a postranslationally modified set of species or as a series of aggregates. It is an acidic protein with a pI of 4.9, and under certain conditions migrates as a discrete band of 11 kDa on SDS-PAGE gels. The carbohydrate structures are thought to consist of O-linked GalNac (Jardim *et al.*, 1995a). The gene for KMP-11 has been cloned and reveals a single open reading frame of 276 bp. There are 3 copies of the gene; however, they are not tandemly repeated. The molecule has 86% α-helical content, and probably consists of a helix loop helix motif. One third of the α-helices are amphiphilic suggesting areas for insertion into the cell membrane (Jardim *et al.*, 1995b).

The function of KMP-11 in the biology of *Leishmania* is unknown. It is known; however, that KMP-11 induces a strong T-cell response in mice and humans. This response was initially attributed to the carbohydrate portion of the LPG molecule (Jardim *et al.*, 1991). The response to KMP-11 is not H-2 restricted as 7 different strains of mice with different haplotypes all respond to this antigen. Both promastigote and amastigote primed lymphocytes respond to stimulation with KMP-11 by production of IL-2. Therefore, this molecule may be useful for vaccine development against *Leishmania* (Tolson *et al.*, 1994a).

Lipophosphoglycan (LPG):

Another major surface glycoconjugate of *Leishmania* is lipophosphoglycan (LPG). This molecule is shed into the medium, and is also known as the *Leishmania* excretory factor (King *et al.*, 1987). LPG shares the same tripartite structure in all species of *Leishmania*. 1) A backbone of PO_4-6Gal (β1-4)Man α1-repeats. 2) These are linked via a phosphosaccharide core of PO_4-6Gal(α 1-6)Gal(α1-3)Galf(β1-3)[Glc(α1-)PO4-6]-Man(α1-3)Man(α1-4)GlcN(α1-6). 3) The phosphosaccharide core is linked to a *lyso*-1-O-alkylphosphatidylinositol anchor (Turco *et al.*, 1987; Orlandi and Turco, 1987; McConville *et al.*, 1987; Turco *et al.*, 1989).

Unlike gp63, LPG is highly polymorphic between species of *Leishmania* (McConville *et al.*, 1987). Variation occurs in the composition and frequency of the oligosaccharide side chains that branch from the galactose residues of the backbone repeat unit (Turco *et al.*, 1987; Ilg *et al.*, 1992; McConville *et al.*,

1992). Differences may also occur in the mannose-containing oligosaccharides that cap the phosphoglycan chains (McConville *et al.*, 1990; Ilg *et al.*, 1992). These differences are evident using SDS-PAGE: the LPG of *L. major* runs as an elongated band from 5 to 40 kDa (McConville *et al.*, 1987), whereas *L. donovani* runs as a 10 to 20 kDa band (Turco *et al.*, 1987). Distinct antigenic sites occur on LPG between species (Handman *et al.*, 1984a) and within LPG (Kelleher *et al.*, 1994).

LPG is developmentally regulated. Promastigotes in culture undergo a maturation process whereby organisms from the log phase are not infective to macrophages, while the stationary phase promastigotes are highly infectious. Stationary phase promastigotes of *L. major* express LPG as a 62 to 116 kDa band, while log phase promastigotes express a band from 5 to 40 kDa (Sacks and Da Silva, 1987; Sacks *et al.*, 1990; McConville *et al.*, 1992; Turco and Sacks, 1991). The LPG forms a thick coat on the surface of the metacyclic promastigote due to doubling of the number of phosphorylated saccharide units (Pimenta *et al.*, 1991).

LPG is also present on the surface of amastigotes (Schnur *et al.*, 1972, Williams *et al.*, 1986b) and infected macrophages (Berman and Dwyer, 1981; Williams *et al.*, 1986b). The LPG in amastigotes is structurally distinct from promastigote LPG. These distinctions were based on comparing the two stages of *L. major*, since amastigote LPG in *L. donovani* is barely detectable (McConville and Blackwell, 1991). The phosphoglycan moiety is larger, possesses fewer phosphorylated saccharide units and is less anionic than promastigotes. The migration pattern in SDS-PAGE is intermediate between the procyclic and metacyclic promastigotes, it runs as a 40 to 100 kDa band (Turco and Sacks, 1991; Moody *et al.*, 1993). The cap structure at the non-reducing terminus also differs between amastigote and promastigote stage (Moody *et al.*, 1993). Not surprisingly, antigenic differences occur between the two stages (Glaser *et al.*, 1991). A major difference between the two stages is the level of expression of the molecule: 5×10^6 copies of LPG in promastigotes (McConville and Bacic, 1989), whereas only 6×10^4 copies are expressed in amastigotes (Moody *et al.*, 1993). Amastigotes LPG is located on the surface and flagellar pocket (Williams *et al.*, 1986b; Stierhof *et al.*, 1991; Pimenta *et al.*, 1991).

Several roles for LPG have been described within the mammalian host. Binding of LPG directly to the CR3 receptor will be discussed in detail later in

this chapter. LPG triggers complement activation for C3 deposition. The elongated length of the side chains in the metacyclic promastigotes protect the parasite from complement-mediated lysis (Franke *et al.*, 1985; Puentes *et al.*, 1988; Puentes *et al.*, 1991). An alternative mechanism of complement activation may occur through the mannan-binding protein (MBP) that binds to LPG (Green *et al.*, 1994). MBP, found in the serum, is a known activator of complement.

LPG appears to play a role in survival of the parasite inside macrophages, because LPG deficient mutants do not survive in the host cell (Handman *et al.*, 1986; McNeely and Turco, 1990; Frankenburg *et al.*, 1992). This may occur through direct effects of LPG on macrophages. It inhibits macrophage chemotaxis (Frankenburg *et al.*, 1992), respiratory burst activity (McNeely and Turco, 1987), and intracellular signaling through the protein kinase C pathway (McNeely *et al.*, 1989; Descoteaux *et al.*, 1991; 1992).

LPG as a vaccine candidate has also been studied. Partial protection was obtained with purified LPG (Handman and Mitchell, 1985). Better protection was obtained when LPG and *Cornybacterium parvum* were incorporated into liposomes, 5/6 animals remained uninfected (McConville *et al.*, 1987). Without *C. parvum*, LPG in liposomes was not protective. A combination of LPG and gp63 incorporated into liposomes partially protected against infection, 3/5 mice were uninfected (Russell and Alexander, 1988).

LPG plays an important role in attachment of the promastigote to epithelial cells in the midgut of the sandfly through lectin-like interactions (Pimenta *et al.*, 1992). Interestingly, only the procyclic stage attaches to epithelial cells. The modification in the structure of LPG in the metacyclic stage allows the parasites to detach in preparation for entry into the vertebrate host (Pimenta *et al.*, 1992; Sacks *et al.*, 1995). Attachment to epithelial cells is dependent on the species of sandfly vector and species of *Leishmania*. Thus, it is one mechanism that results in the specificity of *Leishmania* species for certain species of sandfly and is a possible evolutionary selection pressure for the high interspecies variability of this glycolipid (Pimenta *et al.*, 1994). See Sacks *et al.* (1994) for a review.

Glycoinositolphospholipids (GIPL):

A group of surface glycolipids, amphipathic glycoconjugates that are shed into the medium by promastigotes of *L. major*, are the glyco-inositol-

phospholipid (GIPL) (McConville and Bacic, 1989). Although GIPLs show some structural homology with the membrane anchors of LPG and gp63 (McConville and Bacic, 1989) they are distinct glycolipids. MAbs that recognize GIPL do not react with LPG (Handman and Hocking, 1982). Further evidence that this molecule is different from LPG is that antisera from mice infected with the LRC-L119 strain of *L. major* which lack LPG, recognize GIPL but not LPG.

GIPLs consist of a linear glycan with a core sequence of Gal$_f$(α1-3)Man$_p$(α1-3)Man$_p$(α1-4)GlcN$_p$(α1-6)myo-inositol. This core is attached to an alkylacyl-phosphoinositol or a lyso alkyl-phosphoinositol (McConville *et al.*, 1990a). At least six GIPLs have been described for *L. major*, that are heterogeneous in both their lipid and carbohydrate moieties. The core structure is conserved among different species of *Leishmania* (McConville *et al.*, 1990a; McConville and Blackwell, 1991; Winter *et al.*, 1994). There are also shared epitopes; mAbs that recognize GIPL of *L. major* cross-react with *L. donovani* and *L. mexicana* (Handman and Hocking, 1982). Differences occur in the presence and composition of oligosaccharide branches from the core and in which GIPLs are predominantly expressed (McConville *et al.*, 1990a; McConville and Blackwell, 1991; Winter *et al.*, 1994). Certain GIPLs may be precursors of LPG since there is a large intracellular pool of GIPLs (McConville and Bacic, 1989); however, others are independent molecules that are expressed on the surface (McConville and Bacic, 1990).

GIPL is abundant in the promastigotes, amastigotes and on the surface of infected macrophages (Elhay *et al.*, 1988). The level of expression in *L. major* is 1×10^7 molecules/cell for promastigotes and it is estimated to be 20% of surface expressed molecules (McConville and Bacic, 1990). In the amastigote stage there are 6×10^6 molecules/cell and it has been proposed that the GIPLs of amastigotes may coat 45-60% of the amastigote surface (Schneider *et al.*, 1993). These molecules differ between the two stages of parasite. Amastigote GIPLs have shorter glycan chains and express more of the lyso-alkyl-phosphoinositol forms than the alkylacyl-phosphoinositol forms (Schneider *et al.*, 1992).

To date, no function has been proposed for the GIPLs. It is known that the GIPLs are very antigenic in humans, because high levels of anti-GIPL antibodies occur in *Leishmania* infected individuals (Avila *et al.*, 1991; Rosen

et al., 1989). Their extreme abundance, 10^7 molecules/cell, suggests they play an important role in the biology of both stages of the parasite.

Evidence that entry involves receptor/ligand binding:

Entry of most parasitic protozoa into macrophages occurs in two main stages. First, the parasites must attach to the cell surface, presumably by receptor/ligand interaction, and second, they must be enclosed into the phagosome and internalized (Blackwell and Alexander, 1983). These processes require that both host cell and the parasite are physiologically intact and functioning.

The need for a physiologically intact host cell is evident from the following studies. Treatment of macrophages with iodacetamide, a metabolic inhibitor of glycolysis, reduces *L. tropica* promastigote binding by 70% (Zenian, 1981). In contrast, *L. mexicana* amastigote binding is unaffected by iodacetamide treatment, whereas internalization is significantly reduced (Benolial *et al*, 1980). Cytochalasin B, an inhibitor of phagocytosis, significantly reduces the internalization of promastigotes (Chang, 1979; Alexander, 1975; Aikawa *et al.*, 1982) and amastigotes (Wyler and Suzuki, 1983) of different *Leishmania*. Treatment of macrophages with proteases and glycosidases, such as trypsin, pronase, neuraminidase and α-mannosidase, also significantly reduces binding and/or internalization of *Leishmania* (Benoliel *et al.*, 1980; Chang, 1981; Wyler and Suzuki, 1983). It should be pointed out that only certain proteases and glycosidases are effective on certain species of *Leishmania*.

Active parasites also appear to be necessary for binding and entry. Decreased binding and internalization results when promastigotes or amastigotes are treated with cytochalasin B, or a variety of proteases and glycosidases (Chang, 1981; Aikawa *et al.*, 1982; Wyler and Suzuki, 1983). Interestingly, β-galactosidase treatment of amastigotes of *L. tropica* results in enhanced binding, possibly due to exposure of glucose and mannose residues which mediate attachment via monosaccharide residues (Wyler and Suzuki, 1983).

Significantly fewer dead promastigotes of *L. donovani* are internalized by macrophages when compared to live parasites (Chang, 1979). In contrast, Zenian *et al.* (1979) reported that glutaraldehyde-fixed promastigotes of *L. tropica* are internalized by macrophages at the same rate as live parasites, but

the parasites are not engulfed by glutaraldehyde-treated macrophages (Zenian *et al.*, 1979).

Binding and entry of *Leishmania* is also temperature dependent. At 4°C, binding of *L. donovani*, *L. tropica* and *L. mexicana* promastigotes is reduced and internalization is abolished (Chang, 1981; Zenian, 1979; Merino *et al.*, 1977; Benoliel *et al.*, 1980; Wyler, 1982). *L. tropica* promastigotes bind equally well at 4°C or 37°C to plasma membrane vesicles isolated from macrophages (Klempner *et al.*, 1983). Ethylene diamine tetraacetic acid (EDTA) and ethyleneglycoltetraacetic acid (EGTA) inhibit attachment of *L. donovani* and *L. m. mexicana* to macrophages, indicating a need for Ca^{++} and Mg^{++} (Chang, 1981; Bray, 1983).

The process of entry does not appear to be an active penetration on the part of the parasite as neither promastigotes or amastigotes possess recognizable penetration organelles. Moreover, no specific orientation of promastigote prior to entry has been observed by Chang (1979). There are reports; however, that indicate that entry of promastigotes occurs by the anterior end, i.e. flagellum first (Alexander, 1975; Zenian *et al.*, 1979) or posterior end (Akiyama and Haight, 1971). Although the process of parasite entry has not been fully elucidated, it has been suggested that entry into the phagocytic cells may occur according to the zipper hypothesis involving a sequential and circumferential interaction between phagocyte receptors and ligand on the surface of the parasite (Silverstein, 1977).

The results of the above studies indicate that entry of *Leishmania* into macrophages involves receptor/ligand binding and that promastigote and amastigote stages of the parasite may use different receptors for entry into macrophages. Moreover, differences in the mechanisms of entry appear to exist among species of *Leishmania*.

Receptors utilized in binding and internalization:

Several receptors on the macrophage surface participate in phagocytosis of the parasite. The parasite has taken advantage of the mechanisms employed by the host to engulf particles, which normally results in destruction of the invasive particle. Interestingly, *Leishmania* have evolved to resist destruction after entry into the macrophage and therefore

they 'encourage' the host cell to engulf them. The following is a review of the different macrophage receptors.

Mannose fucose receptor (MFR):

Dose-dependent inhibition of *L. donovani* entry into macrophages occurs after addition of a number of different monosaccharides (Channon *et al.*, 1984; Chang, 1981). A receptor that is used for entry by promastigotes is the mannose/fucose receptor (MFR) which is specific for L-fucose, D-mannose, N-acetyl-D-glucosamine and D-glucose. Inhibition assays using mannan (Channon *et al.*, 1984) and mannan-BSA (Wilson and Pearson, 1986) demonstrated that *L. donovani* promastigote entry into macrophages is inhibited by 40% and 56%, respectively. A 97% inhibition occurs in macrophages adhered to mannan-coated coverslips prior to addition of promastigotes (Wilson and Pearson, 1988). In this system, macrophages adhered to mannan-coated coverslips were capable of phagocytosing IgG-coated sheep red blood cells, indicating that the Fc receptor was not altered by down-modulation of the MFR (Wilson and Pearson, 1986). In addition, intravenous administration of mannan and several neoglycoproteins such as N-acetylglucosamine-BSA, prior to infection in hamsters, inhibits the clearance of *L. donovani* promastigotes from the blood, providing evidence for the relevance of the MFR in vivo (Chakraborty and Das, 1988). However, binding of amastigotes of *L. donovani* to macrophages is not inhibited by soluble mannan (Channon *et al.*, 1984; Wilson and Pearson, 1986) but is inhibited by 60% when macrophages are allowed to adhere to mannan-coated coverslips (Blackwell *et al.*, 1985).

Complement receptors (CR1, CR3, CR4):

Leishmania are capable of activating the complement pathways. *L. donovani* activates the alternate complement pathway and activation by stationary phase promastigotes is greater than that by log phase organisms which, in turn, is greater than the activation induced by amastigotes (Wozencraft and Blackwell, 1987). In some species, such as *L. major*, log phase promastigotes activate the alternate complement pathway, whereas stationary phase organisms activate the classical complement pathway (Puentes *et al.*, 1988). Fragments of C3 have been detected on the surface of *L. mexicana* and *L. tropica* , after incubation in normal guinea pig serum

(NGPS), by indirect immunofluorescence using anti-C3 antibody. A concentration of 25% NGPS lysed promastigotes, whereas 4% NGPS enhanced promastigote binding to macrophages (Mosser and Edelson, 1984; Blackwell, *et al.*, 1985); however, NGPS did not enhance amastigote uptake (Blackwell *et al.*, 1985). Promastigotes rapidly accumulate C3b on their surface when incubated in NGPS, and this is converted to C3bi, the inactive form of C3b (Mosser and Edelson, 1985). Electron microscopy using specific anti-C3 mAbs followed by gold-labeled protein-A revealed C3 deposition on promastigotes and amastigotes of *L. donovani* (Wozencraft *et al.*, 1986). Russell (1987) estimated there are 8 -12 x 10^5 C3 molecules bound to the promastigote surface. In addition, they reported that greater than 80% of C3 on the surface is in the form of C3b not C3bi. A recent study suggests activation of the complement cascade for deposition of C3 on the parasite surface may occur through the mannose binding protein (MBP). This serum protein binds to purified LPG, and is a known activator of the complement cascade (Green *et al.*, 1994).

An interesting method used by *Leishmania* to gain entry into macrophages is through the macrophage complement receptors. Promastigotes bind complement components on their surface, the protein complexes bind to the macrophage complement receptors CR1 or CR3 and the parasite ent the host cell (Mosser and Edelson, 1984; 1987). Macrophage complement receptors involved in binding C3 on promastigotes have been identified using anti-receptor mAbs. MAb M1/70, also known as anti-Mac-1, which recognizes CR3, inhibits *L. donovani* promastigote entry into macrophages in the absence of serum, but it has very little effect on amastigote entry (Blackwell *et al.*, 1985). MAb M1/70 similarly decreases *L. major* promastigote entry into macrophages and a greater degree of inhibition occurs in the presence rather than absence of normal guinea pig serum (NGPS) (Mosser and Edelson, 1985). Coverslips coated with mAb M1/70 inhibited 80% of promastigotes from entering macrophages adherent to these coverslips (Blackwell *et al.*, 1985). Interestingly, when mouse resident peritoneal macrophages were allowed to adhere to coverslips coated with both mannan and mAb M1/70 no additive effect on inhibition of *L. donovani* entry occurred (Blackwell *et al.*, 1985). In contrast, there is a synergistic inhibition of *L. donovani chagasi* attachment to human peripheral blood monocyte-derived macrophages when the macrophages are incubated on

mannan coated coverslips and exposed to anti-Mac-1 mAb (Wilson and Pearson, 1988). Increasing inhibition by mAb M1/70 occurs as promastigotes of *L. donovani* and *L. major* develop from the non-infective to infective stage, further demonstrating the importance of complement receptors in entry (Cooper *et al.*, 1988).

Da Silva *et al.* (1989) demonstrated differences in binding between log and stationary phase promastigotes of *L. major* to human macrophages. Log phase promastigotes bound to macrophages in the absence of fresh serum, whereas stationary phase promastigotes did not. They also showed, using a series of mAbs against the macrophage complement receptors CR1 and CR3, that log phase promastigotes bind the CR3, whereas stationary phase organisms bind the CR1. Blocking of the CR1 resulted in only a 50% decrease in stationary phase promastigote binding to macrophages, indicating the involvement of yet other macrophage receptor(s) for parasite entry. Mosser *et al.* (1992) also demonstrated the need for opsonization with C3 in promastigote binding to the CR3, by showing that in the presence of fresh serum more promastigotes bind to COS cells transfected with the CR3 than the mock-transfected cells that do not express this receptor. These studies demonstrate that there are inter-species differences in the complement receptors utilized by *Leishmania*, nevertheless, it appears that all *Leishmania* employ complement receptors as one means of entry.

Two studies have examined the use of the CR3 in amastigote entry into macrophages. A 14-40% decrease of amastigotes of *L. donovani* into resident peritoneal cells was observed in the presence of an anti-CR3 mAb (Blackwell *et al.*, 1985). However, a 60% reduction in entry was observed when the macrophages were adhered to glass slides coated with the mAb. Blank *et al.* (1993) reported an 80% inhibition of entry of amastigotes of L. major into Langerhan cells in the presence of the same anti-CR3 mAb. Hence it would appear that the CR3 is also used for entry of *L. major*.

Advanced glycosylation endproduct receptor (AGE-R):

The macrophage receptor for advanced glycosylation endproducts (AGE) has also been implicated in binding of promastigotes to macrophages. AGE products arise from time dependent non-enzymatic reactions of glucose with proteins. AGE-BSA blocks *L. major* promastigote entry into macrophages in the order of 50%, while BSA alone had no inhibitory effect

(Mosser *et al.*, 1987). Binding and uptake of C3bi-coated red blood cells and zymosan were unaffected when macrophages were pre-incubated with AGE-BSA indicating that separate receptor systems are involved. Simultaneous addition of AGE-BSA and anti-Mac-1 resulted in a 90% inhibition of promastigote entry, suggesting that both receptors for AGE, and complement CR3 receptor play a crucial role in promastigote entry (Mosser *et al.*, 1987).

Fibronectin receptor (FnR):

Fibronectin receptors on macrophages have also been reported to be involved in binding and entry of promastigote into their host cell. Anti-fibronectin antibodies, and their $(Fab')_2$ fragments block the binding of promastigotes and amastigotes of *L. m. amazonensis* and *L. major*, to human peripheral blood monocytes (Wyler *et al.*, 1985; Rizvi *et al.*, 1988). Promastigotes cultured in medium lacking fibronectin bind and infect fewer human peripheral blood monocytes than promastigotes grown in medium containing fibronectin. Promastigotes and amastigotes preferentially bind to fibronectin coated coverslips, and fibronectin binds to the promastigote surface (Wyler *et al.*, 1985). Anti-fibronectin mAbs and polyclonal sera can precipitate the gp63 molecule from ^{125}I-labeled or ^{35}S-methionine labeled promastigotes of *L. donovani chagasi* indicating sequence homology between gp63 and fibronectin (Rizvi *et al.*, 1988). It appears that fibronectin on the parasite surface binds to the fibronectin receptor on macrophages because pre-treatment of macrophages with anti-fibronectin antibodies does not inhibit parasite binding (Wyler *et al.*, 1985). Incubation of macrophages with synthetic peptides of the RGDS sequence (a sequence recognized by the fibronectin receptor) results in a decrease (50%) in the attachment of promastigotes to macrophages (Rizvi *et al.*, 1988). Initially, Button and McMaster (1988) reported that gp63 contains an RGD sequence similar to that found in fibronectin. Further studies revealed no such sequence and the occurrence of RGD was reported to be an error in sequencing (Button and McMaster, 1990; Miller *et al.*, 1990). However, another sequence in gp63, ser-arg-tyr-asp (SRYD), mimics the RGD sequence (Soteriadou *et al.*, 1992) and antibodies against the RGD sequence of fibronectin bind to SRYD. A 71% inhibition in binding of promastigotes to macrophages occurs in the presence of an octapeptide containing the SRYD sequence. It is therefore possible that promastigotes may bind directly to the macrophage fibronectin receptor.

Fc receptor (FcR):

The Fc receptor on macrophages may also be a candidate receptor for promastigote entry, since specific anti-parasite antibodies are common during both acute and chronic stages of this infectious disease. Entry of *L. donovani* promastigotes almost doubles when promastigotes are opsonized with sub-agglutinating titers of anti-*Leishmania* serum. Treatment of opsonized promastigotes with Protein-A, or macrophages with anti-*Leishmania* serum, reduces binding up to 50% (Chang, 1981). Internalization of gp63-coated beads is enhanced in the presence of anti-gp63 antibodies, suggesting Fc receptor involvement (Russell *et al.*, 1989). However, Blackwell *et al.* (1985) reported no inhibition of *L. donovani* promastigote or amastigote binding to macrophages adherent to coverslips coated with a mAb against the IgG_{2b}/IgG_1 Fc receptor.

Parasite ligands utilized in binding and internalization:

Fifteen years ago, Benoliel *et al.* (1980) reported that a crude extract of *L. mexicana* inhibited parasite entry into macrophages. One parasite molecule implicated in binding macrophages is the surface protease gp63. This has been demonstrated in several ways. Fab fragments of anti-gp63 antibodies, produced by immunizing animals with chromatography purified gp63, decrease binding of *L. mexicana* promastigotes to macrophages by 65-70%. In addition, proteoliposomes coated with purified gp63 are avidly phagocytosed by macrophages and anti-gp63 antibodies or mannan can almost completely block phagocytosis of the gp63-coated liposomes (Russell and Wilhelm, 1986). Similarly, immunoaffinity purified gp63 from *L. m. amazonensis* promastigotes inhibits binding of parasites to macrophages by 50% (Chang and Chang, 1986) and gp63 from *L. donovani chagasi* was also shown to inhibit promastigote binding to macrophages (Wilson and Hardin, 1988). The surface molecule LPG is also involved in parasite binding. Fab fragments of an anti-LPG mAb, reduced binding of promastigotes of *L. major* to macrophages by 80% (Handman and Goding, 1985). The epitope recognized by the anti-LPG is on one of the phosphorylated oligosaccharide repeats of LPG and is unique to *L. major* (Kelleher *et al.*, 1992).

The results of experiments using purified gp63 and LPG coupled to beads indicate that both molecules participate in binding and entry of parasites into macrophages. Gp63-coated beads attached to macrophages but

were not phagocytosed, whereas those coated with both gp63 and LPG were engulfed by macrophages. In addition, a synergistic inhibition of promastigote entry into macrophages is evident when macrophages are incubated with soluble gp63 and LPG. This occurs with promastigotes of *L. mexicana*, *L. donovani*, and *L. major* (Russell and Wright, 1988).

A recent study demonstrated that the binding of amastigotes of *L. major* is inhibited by purified LPG. An 80% reduction in binding was observed with LPG purified from amastigotes (Kelleher *et al.*, 1995). It appears, therefore, that the LPG on amastigotes binds directly to macrophages. The phosphorylated oligosaccharide on LPG of promastigotes that binds to macrophages (Kelleher *et al.*, 1992) is not used in binding amastigotes. Instead it appears that a site within the conserved regions of LPG is used since LPG from other *Leishmania* species blocks the binding of amastigotes of *L. major* to macrophages (Kelleher *et al.*, 1995).

Debate over which complement receptor is utilized in *Leishmania* entry is on going. The macrophage receptor CR3 that binds to complement component C3bi, recognizes an RGD peptide sequence. A synthetic peptide containing the gp63 RGD sequence (pep63) was shown to inhibit the binding of gp63-coated beads to macrophages (Russell and Wright, 1988) In addition, pep63 inhibited sheep erythrocytes coated with C3bi from entering macrophages (Russell *et al.*, 1989). Anti-pep63 antibodies recognize both gp63 and the α-chain of human C3 in immunoblots indicating structural similarities between RGD of gp63 and C3. Fab fragments of anti-pep63 inhibit the binding of gp63-coated beads to macrophages. However, Fab fragments did not inhibit sheep erythrocytes coated with C3bi from binding to macrophages, suggesting that the antibody probably recognized denatured C3 and not C3 in its native form (Russell, *et al.*, 1989). Another synthetic peptide sequence, KQQAGDV, a sequence in fibrinogen which is recognized by CR3, inhibits gp63-coated beads from binding to macrophages (Russell and Wright, 1988). Macrophages cultured on mAb OKM10-coated coverslips bound less gp63-coated beads when compared to cells not cultured on mAb-coated coverslips. OKM10 recognizes the C3bi site on CR3. Another mAb, IB4, which recognizes the β-chain of CR3 also inhibited gp63-coated beads from attaching to macrophages. From these results Russell and Wright (1988), concluded that gp63 contains a sequence that binds directly to the CR3 at the identical site as the C3bi. Cooper *et al.* (1988); however, argued that stationary phase

promastigotes bind CR3 by a site distinct from C3bi, possibly via a lectin-like site. This is based on the observation that stationary phase promastigotes are not inhibited by sodium salicyl hydroxamate (SAHA) nor by a mAb 5C6, both of which block the C3bi binding site. Further evidence for a distinct binding site is that the anti-CR3 mAb OKM1, which is believed to recognize the lectin-like site on CR3, was shown to inhibit *L. donovani chagasi* ingestion by human monocyte-derived macrophages. Whereas mAb OKM10, which recognizes the C3bi site of CR3, does not inhibit promastigote attachment (Wilson and Pearson, 1988).

Gp63 and LPG may also be involved in entry into macrophages indirectly, through binding of host complement components. The promastigote protease, gp63, has been implicated in forming a complex with C3 on the promastigote surface. Anti-C3 antibodies precipitate the 63 kDa molecule from radiolabeled promastigotes of *L. mexicana*, whereas the other major surface molecule, LPG, is not precipitated. Further evidence for this is that ^{125}I-labeled C3 can be detected on liposomes containing gp63 but not on liposomes containing LPG (Russell, 1987). In contrast, Puentes *et al.* (1988) report that the principle acceptor of C3 on *L. major* promastigotes is not gp63 but LPG. When ^{125}I-labeled C3 is reacted with log and stationary phase promastigotes, anti-gp63 serum precipitated less than 11% of ^{125}I-C3, whereas a mAb which recognized LPG precipitated more than 80% of ^{125}I-C3.

The parasite ligand, FML, blocks entry of the promastigotes and amastigotes of *L. donovani* into macrophages (Palatnik *et al.*, 1989; Palatnik-de-Sousa, 1993). Possibly through interaction with the MFR.

Another parasite ligand, the glycosphinolipid (GSL) of *L. (L.) amazonensis*, causes a four fold decrease in uptake of amastigotes after a 4 hour exposure to macrophages in the presence of 4 μg of GSL. In addition, anti-GSL mAbs reduce the uptake of amastigotes into macrophages (Barbiéri *et al.*, 1993; Straus *et al.*, 1993). The authors concluded in this study that the glycoshingolipid was of parasite origin; however host glycosphingolipids have been described on the surface of amastigotes (McConville and Blackwell, 1991).

SUMMARY & RATIONALE

It is clear from the literature that there are differences in the surface molecules of different species of *Leishmania* and differences in the mechanisms of entry into their host cell. The available evidence indicates that several receptors are employed by the promastigote stage of *Leishmania* to enter the macrophages. It is interesting that *Leishmania* have evolved to exploit the functions of macrophages, especially the use of the complement and Fc receptors normally involved in defense against pathogens. The evidence also suggests that additional, as yet unidentified receptors, are utilized by the parasite to enter its host cell.

Most of the research in elucidating the receptor/ligand interactions in leishmaniasis, focused on the promastigote rather than amastigote interactions with the macrophage. My working hypothesis was that the promastigote and amastigote stage of the obligate intracellular parasite *L. major* use different mechanisms to enter their host cell, the macrophage. This is because the two stages are morphologically dissimilar, they occur in very different environments and their roles in infection are distinct: the promastigote initiates infection in the vertebrate host, whereas the amastigote causes disease.

A number of questions arose from the basic hypothesis that the two stages use different mechanisms for entry:

1) Is there a difference in the uptake of promastigotes and amastigotes into macrophages?

2). Are there differences in receptors used for entry of promastigotes and amastigotes into macrophages?

3) Are there differences in the surface composition and antigenic structure of amastigotes and promastigotes?

4) Are there unidentified surface molecule(s) involved in the phagocytosis of amastigotes?

To help in addressing these questions I also studied two sources of amastigotes: the standard source obtained from BALB/c mice and another source from *scid* mice. The *scid*-source amastigotes were considered a 'cleaner' source due to the lack of host IgG on its surface. These two sources were compared to determine whether the presence of different host

components on the amastigote stage altered the mechanisms for entry into the macrophage.

These questions were addressed in this thesis to determine whether different mechanisms are involved in the entry of promastigotes and amastigotes of *L. major*. An understanding of the mechanisms of transfer of amastigotes from macrophage to macrophage should provide a means of blocking the entry of disseminating parasites into its host cell, thereby reducing the severity of this devastating infectious disease.

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Chapter II: General Materials and Methods.

Animals:

Six to 10 week old, male, specific pathogen-free BALB/c mice (Charles River, St. Constant, Que.) were used to maintain *L. major*. Mice were housed in 0.2 µm filter top cages and food and water were given ad libitum. *Scid* mice (C.B17/Icr-*scid*) were initially purchased from Taconic Laboratories (German Town, NY). A *scid* mouse breeding colony was established in our animal facility. Breeding pairs were made by brother-sister pairing of mice at six weeks of age. The *scid* mice were maintained under sterile conditions, whereby, their cages, food and water were autoclaved and the mice were handled in a barrier flow hood. Sulfatrim (Apotex, Toronto, Ont.) was added to their water during the first two weeks following weaning, at a concentration of 40 mg of sulfamethoxazole and 8 mg of trimethoprim /250 ml of water. Six to 10 week old SPF-derived, male C57BL/6 mice (Taconic Laboratories) were used as a source of bone marrow-derived macrophages. These mice were housed in a P2 barrier facility and routinely tested for bacterial pathogens and sub clinical viral infections.

Parasite:

The NIH 173 (WHOM/Ir/-/173) strain of *L. major* was used unless stated otherwise. This strain of *L. major* was isolated from a patient in Iran (Chang *et al.*, 1986). The other strain of *L. major* that was used for a comparison of rapid versus slow metastasis in vivo, was the Friedlin strain NIH 80 (WHOM/IL/80/FRIEDLIN), which was isolated from a patient in Israel who had multiple lesions (Sacks *et al.*, 1985).

Isolation of amastigotes:

Amastigotes were maintained by serial passage in BALB/c mice (50 µl containing $2x10^6$ amastigotes inoculated subcutaneously into footpads)(Belosevic *et al.*, 1988). Mice were either alert when infected, or anaesthetized when a large number of animals were used to obtain amastigotes for protein purification. METOFANE (Methoxyflurane) (Pitman-Moore inc., Mississauga, Ont.) was the anesthetic employed and 500 µl was added to a 1L beaker with lid for every 5 mice.

Four to 5 weeks after infection, monodispersed amastigotes were obtained by disruption of infected footpad tissue and passage through #50 stainless steel mesh screens into Dulbecco's Modified Culture Medium (DMEM) (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS, Gibco) and gentamicin

(Sigma, St Louis, MO). Parasites were stained with fluorescein diacetate (FDA) (ICN, Cleveland, OH) and ethidium bromide (EB) (ICN) (Jackson *et al*, 1985; Belosevic *et al.*, 1988) and viable amastigotes were counted using a hemocytometer.

Isolation of large numbers of amastigotes for protein purification:

A separate procedure was used to obtain large numbers of amastigotes for protein purification using phosphate buffered saline (PBS) pH 7.2 as the isolation medium. The cell pellet following 200 x *g* centrifugation was re-homogenized by 10 strokes using a Ten Broek homogenizer and the supernatants pooled with that from the first round of homogenization and centrifuged at 800 x *g* for 10 min. The cell pellet was disrupted and re-suspended in 168 mM NH_4Cl for 10 min. to lyse the red blood cells. During this time the cells were rapidly mixed then passaged through a 27 gauge needle to disrupt clumps of parasites and intact macrophages. The amastigotes were washed three times at 800 x *g* in PBS, re-suspended in PBS and stored at -80°C.

Amastigote antigen preparation:

The amastigote antigen preparation used to initiate an inflammatory response in mice, was prepared using amastigotes from *scid* mice. The amastigotes were killed by three cycles of rapid freeze-thaw, and the cells disrupted by sonication, using three 20 second bursts at maximum capacity, with a Kontes (Vineland, NJ) Micro-Ultrasonic Cell Disrupter. The protein content was estimated using the Micro BCA Protein Assay (Pierce, Rockford, IL) and adjusted to 1 mg/50 µl in PBS.

Promastigotes:

Promastigotes were maintained at 24°C by serial passage in Cunningham's medium (Cunningham, 1977) containing 20% heat-inactivated fetal bovine serum (FBS) and 50 µg/ml gentamicin. For enumeration, the promastigotes were stained with FDA-EB, suspended in 0.1% PBS-agar, and counted with a hemocytometer. Promastigotes were harvested when they were in the stationary phase, at a concentration of 4-7 x 10^7/ml after 5 to 6 days of culture (Sacks *et al.*, 1985). Metacyclic promastigotes were obtained by selection with peanut agglutinin (PNA) (Sigma) using the method of DaSilva *et al.* (1987). To obtain large numbers of PNA^- promastigotes the cells were washed 2 times in DMEM and re-suspended in PNA dissolved in DMEM at a concentration of 100 mg/2-5x10^8 promastigotes. The suspension was allowed to sit for 30 min. at RT and then layered onto 50%

FBS/DMEM and left for an additional 30 min. The upper layer containing non-agglutinated promastigotes was collected and washed 2 times before use.

Assessment of infection:

Footpad measurement:

Cutaneous disease was assessed by estimating the change in thickness of the infected footpad of each mouse compared to the non-infected contralateral foot. Enumerations of amastigotes from the footpad, popliteal lymph nodes, liver and spleen were performed by mashing the organs through a stainless steel screen using the plunger of a 10 c.c. syringe, then homogenizing the tissue using a Ten Broek homogenizer. The suspension was centrifuged at 800 x *g*. Weights of both organs were obtained prior to emaceration. The number of live amastigotes was determined using FDA-EB staining.

Limiting dilution assay for enumerating the number of parasites:

The limiting dilution assay (Titus *et al.*, 1985) was used to detect parasites in the first two weeks of infection when parasite numbers were too low for detection using the FDA stained amastigotes method. Briefly, amastigotes obtained from the footpad of infected mice were suspended in DMEM with 10% FBS and a series of 10 fold dilutions were made in test tubes. The amastigote suspensions were added to wells of 96 well plates containing blood agar, made from fresh rabbit serum. The plates were incubated at RT for 4 days after which the wells were examined under an inverted microscope for the presence of promastigotes.

Measurement of inflammation :

Cells from the footpads and lymph nodes, spleen and liver, were isolated in DMEM by disrupting the tissues through a #50 stainless steel screen using the plunger of a 10 c.c syringe. The cell suspension was centrifuged at 300 x *g* for 10 min. and the number of nucleated cells counted with a hemocytometer.

Macrophages:

Bone marrow-derived macrophages:

Bone marrow-derived macrophages (BMDM) were obtained by flushing the femurs of C57BL/6 mice with sterile Dulbecco's phosphate buffered saline (PBS) (Gibco) (Belosevic *et al.*, 1988; 1989). Cells were sedimented by centrifugation at 300 x *g* for 10 min. and re-suspended at a concentration of 1 x 10^6 viable nucleated cells in DMEM with 10% FBS 50 µg/ml gentamicin and 10% L cell-conditioned medium (LCCM) (day 7 supernatants of cultured L-929 cells were used as the source of CSF-1). Bone marrow-derived cells were cultured in 50 ml sterile

polypropylene centrifuge tubes (Corning, Corning, NY) and fed every 4 days with 1.5 ml of L cell-conditioned medium. The cells were used after 9 to 11 days of culture when approximately 98% of the cells are macrophages. To harvest, the cells were placed in ice for 30 min. and centrifuged at 300 x *g* at 4°C for 10 min.

Resident peritoneal macrophages:

Resident peritoneal macrophages were obtained by peritoneal lavage with 10 ml of DMEM medium containing 10% FBS. The peritoneal fluid from 10-20 mice was pooled and samples removed for differential and total cell counts. The cell suspension was centrifuged at 300 x *g* at 4°C for 10 min. Cells were re-suspended at $5x10^5$ macrophages/ml in DMEM with 10% FBS.

Inflammatory peritoneal macrophages:

Inflammatory peritoneal cells were elicited by intraperitoneal injection of 1 ml sterile 3% thioglycolate (Difco, Detroit, MI) (Stevenson *et al.*, 1981; Belosevic and Faubert, 1986). The cells were harvested by peritoneal lavage 4 days after thioglycollate injection.

Macrophage cell lines:

The macrophage cell lines were maintained in vitro by serial passage in DMEM containing 10% FBS and 50 µg/ml gentamicin. The medium in flasks containing confluent cells was decanted, replaced with fresh medium and the cells dislodged using a cell scraper (Costar, Cambridge, MA). The macrophage cell lines Ba-1 and Ana-1 were a kind gift of Dr. Danuta Radzioch (Host Resistance Program, Montreal General Hospital Research Institute, Montreal, Que.). The M-1 and Wehi-3 cell lines were kindly donated by Dr. Larry Guilbert (Department of Immunology, University of Alberta, Edmonton, AB). The J774A.1 and P388D1 macrophage lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). See appendix II for a list of the mouse strains and methods used to produce the cell lines.

Reagents:

Chemicals used to block entry of amastigotes:

The following carbohydrates were tested: N-acetyl glucosamine, L-fucose, mannan (from *Saccharomyces cervisiae*), mannose, ß-glucan (from barley), glucose and maltose (Sigma). The carbohydrates were diluted in DMEM. Complement receptor blocking was examined using the following reagents: nocodazole (NOC) and 1-oleoyl-2-acetyl-sn-glycerol (OAG) were purchased from Calbiochem (LaJolla, CA), and phorbol myristate (PMA) (Sigma). The chemicals were dissolved

in dimethylsulfoxide (DMSO) (BDH, Toronto, Ont.) and diluted in medium before use.

Monoclonal Antibodies:

The murine monoclonal antibody (mAb) (DB-1), prepared against rat IFNγ that cross-reacts with mouse IFNγ, was a kind gift from Dr. Peter Van der Meide (TNO Primate Center, Rijswiik, The Netherlands). This antibody was previously used in vivo for neutralization of murine IFNγ (Belosevic *et al.*, 1989). A control mAb of the same IgG isotype, prepared against digoxin was kindly provided by Dr. Ted Hall (Department of Immunology, Walter Reed Army Institute of Research, Washington, DC). Recombinant IFNγ was obtained from Genzyme (Cambridge, MA), Genentech (San Francisco, CA), or as a kind gift of Dr. David Finbloom (Cytokine Research, FDA, Bethesda, MD).

Monoclonal antibodies (mAb) M18/2 and M1/70 against the ß-chain and α-chains of the CR3, respectively, were purchased as cell lines from ATCC. MAb PGP-1 was a kind gift from Dr. Tim Mossman (Dept. Immunology, University of Alberta, Edmonton, AB). MAbs were purified from hybridoma supernatant by ammonium sulfate precipitation and S-200 gel chromatography. MAb 2.4G2 against the FcγRII was obtained from the cell line (ATCC) and antibodies from culture supernatants were purified by ammonium sulfate precipitation. Anti-KMP-11 mAbs L98 and L157 as well as purified KMP-11 were a kind gift of Dr. Terry Pearson (Dept. Biochemistry and Microbiology, University of Victoria, Victoria, BC).

Immune sera from mice:

Parasite-specific IgG was prepared from the serum of BALB/c mice obtained 28-35 days post infection with *L. major*. Blood was obtained by cardiac puncture of mice anaesthetized by I.P. injection of 0.5 ml sodium nembutal (mg/ml) (Abbott Labs, Toronto, Ont). The blood was allowed to clot for one hr at RT then clarified by centrifugation at 13,000 x *g* for 10 min. and stored at -80°C. IgG was isolated by chromatography on a Protein A-agarose column (Sigma). Purified IgG was dialyzed against 3 changes of 2L PBS, at 4°C, using a 25 kDa MW cut off membrane (Spectrum, Houston, TX) and stored at -80°C. The normal mouse IgG, used as a control, was obtained from Sigma.

Immune sera from rabbits:

New Zealand white rabbits were used for polyclonal antibody production. Immune sera against the whole parasite, promastigotes, and amastigotes obtained from BALB/c or *scid* mice, were prepared by repeated injections of 5 x

10^8 dead cells. The anti-integral membrane serum was produced by repeated injections of 100 µg of protein, obtained by Triton X-114 solubilization of *scid* source amastigotes. See the appendix #I for details on the injection schedules used to prepare the rabbit polyclonal antibodies.

Immune rabbit polyclonal antibodies were purified by CM-affigel blue (Bio-Rad, Richmond, CA) followed by ammonium sulfate precipitation, then dialyzed against PBS. $(Fab)_2'$ fragments of rabbit IgG were prepared by pepsin digestion. Six milligrams of IgG were dialyzed against 0.1M sodium acetate for 3 hours. The pH was adjusted to 4.5 with acetic acid and the volume adjusted to 1.25 mg/ml. A total of 140 µg of pepsin (Fisher) was added and the solution incubated for 20 hr at 37°C. The solution was centrifuged at 13,000 x *g* and the supernatant was adjusted to pH 7.2 and dialyzed against PBS. The fragments were purified by FPLC using a Superose 12 gel filtration column. Chromopure rabbit IgG, $F(ab')_2$ fragments were used as a control (Jackson Immunoresearch, West Grove, PA).

Bioassay for Entry of *L. major* into Macrophages:

Cell suspension assay:

Macrophages were suspended in DMEM (Gibco) at a concentration of 2.5 x 10^5 cells / 0.5 ml in polypropylene tubes (Falcon, Becton Dickinson, Lincoln Park, NJ) and incubated for 2 hr at 37°C and 5% CO_2 before use. Test solutions were added (0.1 ml) and the cells incubated for 30 min. at 37°C, after which $5x10^5$ amastigotes or $2.5x10^6$ promastigotes in 0.1 ml were added to the macrophages and incubated for 1 to 2 hr at 37°C. To study the effects of parasite specific IgG on entry, promastigotes and amastigotes were incubated for 30 min. in parasite-specific or normal IgG. Excess antibody was removed by centrifugation for 10 min. at 300 x *g*. The parasites were re-suspended in DMEM and incubated with macrophages for 2 hours. Following incubation the cell suspension was centrifuged at 100 x *g* for 7 min., the supernatant gently removed by aspiration, and the pellet re-suspended in 0.5 ml of DMEM. Cell smears were prepared using a cytocentrifuge (Shandon, Pittsburgh, PA) and stained with Wright stain (Leukostat, Fisher Sci., Orangeburg, NY). The percentage of infected cells and the number of parasites/200 macrophages was determined by microscopic examination of stained cell smears under oil immersion.

The data were presented in 3 forms. 1) The percentage infection: the percent of macrophages out of 200 cells that had ingested one or more parasites. 2) The number of amastigotes per macrophage: the number of amastigotes contained within 100 infected macrophages.

3) The percent reduction of infection:

$$\frac{\text{\% infected control macrophages - \% infected treated macrophage}}{\text{\% infected control macrophages}}$$

Cell adherent assay:

Macrophages were added to wells of eight-well Lab-Tek tissue culture chamber slides (Nunc, Naperville, IL) that were coated with 0.1 mg/ml of poly-L-lysine (molecular weight 70,000 to 150,000; Sigma) for 1 hr at RT. Macrophages were allowed to adhere for 1 hr, and then the wells were washed twice before addition of amastigotes. After 1 hr incubation the wells were washed twice, air dried, fixed and stained, as described above. Slides were coated with immune complexes by the following method: poly-l-lysine coated wells were incubated for 30 min at room temperature with 0.1 ml of a 16.8 mg/ml concentration of albumin, human-dinitrophenol (DNP-albumin, Sigma) washed, and reacted with 0.1 ml of 200 µg/ml concentration of rabbit anti-DNP (Sigma) for 30 min. at RT. To coat with mAb 2.4G2 against the FcRII, or with the carbohydrate mannan, poly-l-lysine coated wells were incubated with up to 50 µg/ml of mAb or up to 10 mg/ml of mannan for 1 hr at RT. The wells were washed twice with DMEM prior to adding the macrophages.

Intracellular killing:

In the intracellular killing assay, macrophages were exposed to amastigotes and macrophages at a one:one ratio for 2 hr at 37°C, in polypropylene tubes containing $5x10^5$ cells, then IFNγ was added. After 72 hr incubation the percentage of macrophages with intracellular amastigotes were determined from smears made by cytocentrifugation and stained with Leukostat stain (Fisher) (Belosevic *et al.*, 1988).

NO_2^- assay:

NO_2^- production was measured on culture supernatants by the Griess reaction (Green *et al.*, 1991). Briefly, cell cultures that had been incubated for 72 hr were spun at 800 x *g* for 10 min. Fifty µl of the supernatant was removed and added to wells of a 96 well plate (Costar), then 100 µl of 1% sulfanilamide (Sigma) and 100 µl of 0.1% N-(1-napthyl)ethlyenediamine (Sigma) were added to each well. After 5 min., the plates were read in a microtiter plate reader (Bio-Tek, Winooski, VT) at 570 nm. $NaNO_2$ (Sigma) was used as a standard for comparison.

Enzyme-linked immunosorbent assay (ELISA):

The presence of surface bound IgG was detected by ELISA using whole parasites as the source of antigen. The parasites were washed three times in sterile PBS, adjusted to $2x10^6$/ml and 100 µl added to each well of a 96 well plate (Immulon 2; Dynatech, Chantilly, VA). The plates were either kept at 37°C for three hr to allow the parasite to settle and adsorb to the well (alternatively, the plates were centrifuged at 800x *g* for 10 min.), then the supernatant was removed and the plates air dried at room temperature for 15-30 min. Plates were stored at -20°C. Isotypes were determined using the Mouse Typer sub-isotyping panel (Bio-Rad) and rabbit anti mouse IgG-horse radish peroxidase (HRP) at a 1:2,000 dilution (Sigma). The substrate used was 2,2'-azino-di-[3-ethyl-benzthiazoline-6-sulfonic acid] (ABTS, Bio-Rad) and the plates read at 405 nm in a microtiter plate reader. Complement component C3 bound to the parasite surface was estimated by ELISA using rabbit anti-human C3 (Serotec, Cedarlane, Hornby, Ont.) at a dilution of 1:2,000, and detected using goat anti-rabbit IgG-HRP (Bio-Rad). Guinea pig serum (GPS) (Cedarlane) was used as the source of exogenous complement. KMP-11 was detected using anti-KMP-11 mAbs L98 and L157 at a dilution of 1:1000. Rabbit anti-mouse IgG-HRP, at a dilution of 1:2,000, was used to detect binding of the mAbs.

Indirect immunofluorescence assay (IFA):

IFA using live amastigotes was performed on ice to prevent internalization of antibodies. One hundred µl of a cell suspension containing 1×10^7 cells/ml in DMEM were added to an equal volume of antibody diluted in DMEM, and incubated for 1 hr on ice. Following incubation, the solution was washed 2 times at 800 x *g* for 10 min. in DMEM. Fluorescein isothiocyanate (FITC) conjugated goat anti mouse-IgG was added and incubated on ice for 1 hr. The cells were washed 3 times in PBS plus 0.01% evans blue (Fisher) then re-suspended in PBS and kept on ice until examined under the microscope. All FITC conjugated antibodies were purchased from Sigma and used at the recommended concentrations.

IFA using live macrophages was performed in the same manner as for amastigotes, except that $5x10^5$ cells were used per tube. Rat mAbs were detected with goat anti-rat IgG (Sigma).

Ammonium sulfate precipitation:

A saturated solution of ammonium sulfate (BDH) was made at 4°C and the pH adjusted to 7.0 using NaOH. An equal volume of ammonium sulfate was slowly added over 5-10 min. and mixed by inversion, this was performed on ice.

The solution was incubated overnight on a rocker table at 4°C, then centrifuged at 41,000 x *g* for 30 min. at 4°C. The pellet was re-dissolved in a small volume of PBS using a glass rod, then dialyzed over night against 3 changes of PBS.

Protein Estimation:

Bio-Rad micro protein assay:

Proteins were quantified by either the Bio-Rad micro protein assay or the Micro BCA protein assay reagent (Pierce). In the Bio-Rad method, samples were diluted in sample buffer and 200 µl added to wells of a 96 well plate (NUNC, Kamstrup, Denmark) followed by 50 µl of dye reagent. The samples were well mixed allowed to sit for 5 min., then read at 595 nm in an automated micro plate reader. BSA, diluted in the same buffer as the unknown samples, was used to make a standard curve, consisting of the following µg/ml concentrations: 1, 5, 10, 15, and 20.

Pierce micro BCA protein assay:

For the Micro BCA method, serial dilutions of the sample were tested and BSA, used to make a standard curve, was diluted in the same buffer as the unknown samples. The samples were placed in 12x75 mm glass tubes (Fisher) and the dye reagents added. The solutions were mixed by vortex and heated for 1 hr at 60°C. After cooling, 100 µl of each sample was added to wells of a 96 well plate (Nunc) and the plates read in an automated micro plate reader at a wave length of 570 nm.

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE):

SDS-PAGE was performed following the method of Laemlii (1980). Briefly, discontinuous gradient gels were prepared using a gradient former (Bio-Rad). Antigen was diluted 1:4 in 1x sample buffer (or 1:2 in 2x sample buffer when the antigen source was dilute). β-mercaptoethanol (Sigma) was used as the reducing agent. Samples in either non-reducing or reducing sample buffer were heated at 100°C for 5 min., spun at 13,000 x *g* to remove particulates and loaded into wells. Mini gels were run at 100 volts for 10 min., followed by 200 volts until the dye front reached approximately 1 cm from the edge of the gel (approximately 35 min.).

Autoradiographs:

The protein II Xi cell was used to run iodinated amastigote samples. Gels were run for 3-4 hours at 100 mA, then stained in Coomassie blue. After destaining the gels were rinsed in deionized H_2O and dried for 2 hr at 80°C. Gels were placed in a Cronex cassette containing intensifier screens (Dupont) and X-

ray film (X-OMAT AR, Eastman Kodak, Rochester, NY) and exposed at -80°C. Films were developed in an automatic film developer (Fuji).

SDS-PAGE gel staining:

Coomassie staining:

Following electrophoresis the gels were stained in 0.1% Coomassie blue R-250 (Bio-Rad) for 1/2 hr then destained in 40% methanol + 10% ethanol. The procedure was carried out at RT on a clinical rotator (Fisher).

Silver staining:

Silver staining of gels was performed using the Bio-Rad silver staining kit. The procedure was carried out at RT on a clinical rotator (Fisher).

Copper staining:

Copper staining was performed using the Bio-Rad copper staining solution. The procedure was carried out at RT on a clinical rotator (Fisher).

Transfer of proteins from gels to nitrocellulose:

Proteins were transferred form gels to nitrocellulose using the Trans-blot Electrophoretic Transfer Cell (Bio-Rad). A 25 mM Tris / 192 mM glycine / 20% methanol buffer pH 8.3 was used at 4°C and proteins were blotted onto 0.2 µm nitrocellulose paper (Bio-Rad), for 2 hours at 100V.

Immunoblotting:

For immunoblotting, the samples on nitrocellulose were blocked overnight at 4°C in 1% milk TTBS buffer: 0.02M Tris, 0.5M NaCl + 0.05% Tween 20 pH 7.5. Primary antibodies were detected using goat anti-rabbit (or anti-mouse) IgG alkaline phosphatase (Bio-Rad). Bands were visualized using p-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-inodyl phosphate (BCIP).

Avidin detection of biotinylated proteins:

Nitrocellulose strips containing biotinylated proteins were blocked for 30 min in TTBS containing 0.2% glutaraldehyde. Blots were washed and blocked in 1% milk TTBS for 1 hr at RT. Blots were reacted with 1:3,000 Avidin-alkaline phosphatase (Bio-Rad) for 1 hr at RT then reacted with developing buffer containing NBT and BCIP.

Radioiodination of amastigotes:

The surface of live amastigotes was radioiodinated with Iodine^{125} by the iodogenation technique, employing the IODO-BEADS Iodination reagent (Pierce). Amastigotes from *scid* mice were isolated from the footpads of mice and purified using the technique for protein purification. The cells, 5 x 10^9 live cells in

0.5 ml of PBS, were placed on ice until use. Three Iodo beads were washed in PBS, dried on filter paper and placed in a 1.5 ml polypropylene microcentrifuge tube (Fisher). To the beads was added 0.495 ml of PBS and 5 µl of Iodine-125, carrier-free sodium iodide in a sodium hydroxide solution, pH 7-11, from a stock of 100 mCi /ml (Amersham, Oakville, Ont). The solution was mixed then allowed to sit for 5 min. at RT. Following the incubation the tube was placed on ice, the amastigotes added, and incubated for 11 min. on ice with vortexing every 2 min. to maintain the cells in suspension. After 11 min., the suspension was removed to a fresh tube, the beads were washed twice with 100 µl of PBS, and the wash added to the amastigote suspension, then the beads were discarded. To remove free I^{-125}, the amastigote suspension was washed 6 times, in PBS, at 800 x *g* for 7 min. in a Costar micro centrifuge on ice. After the fourth and fifth washes the amastigote suspension was passed through a 27 gauge needle to disrupt clumps of cells. Following washing, the cells were rapidly freeze-thawed, 4 times on dry ice, to disrupt the membrane, then the sample was stored at -80°C until use.

Biotinylation:

Live amastigotes were biotinylated following the procedure of Goodloe-Holland and Luna (1987). Live amastigotes from *scid* mice, isolated from the footpads and purified using the technique for protein purification, were suspended in PBS at a concentration of 1×10^9 cells and placed on ice. Immunopure Sulfo-NHS-Biotin (sulfosuccinimidobiotin, Pierce) was dissolved in PBS just prior to use and added to amastigotes to obtain a final concentration of 4 mg/10 ml. The suspension was incubated for 1 hr on ice on a clinical rotator at 150 rpm. Following incubation the cells were washed 3 times in PBS at 800 x *g* for 10 min. at 4°C. Samples were stored at -80°C until use.

Protein isolation from amastigotes:

Chemicals:

The protease inhibitors were all obtained from Sigma and were used at the following concentrations: 1 µg/ml leupeptin, antipain, and pepstatin A; 5 µg/ml aprotinin; 1 mM phenylmethylsulfonylfluoride (PMSF) and 2.5 mM iodacetamide.

The detergents CHAPS, N-octylglucoside, Triton X-114 and Triton X-100 were all obtained from Boehringer-Mannheim (Mannheim, Germany). Triton X-114 and Triton X-100 were of high grade for use in membrane isolation .

Isolation of water soluble molecules:

Amastigotes from *scid* mice were isolated from the footpads of mice and purified using the technique for protein purification. The amastigotes were killed by three cycles of rapid freeze-thaw, and the cells disrupted by sonication, using three 20 second bursts at maximum capacity, with a Kontes (Vineland, NJ) Micro-Ultrasonic Cell Disrupter. The volume of sonicated amastigotes was adjusted to 9 ml /$1x10^{11}$ cells using PBS containing protease inhibitors, the suspension was placed into 13x51 mm polyallomer centrifuge tubes ((Beckman, Palo Alto, CA), and centrifuged at 100,000 x *g* for 1 hr at 4°C. After centrifugation, the lipid plug at the top of the supernatant was carefully removed and the remainder of the supernatant was filtered through a 0.22 µm filter (Millipore, Mississauga, Ont.) and stored at -80°C until use.

The water soluble fraction of amastigotes was concentrated by centrifugation at 800 x *g* at 4°C using CF 25 filter cones (Amicon, Danvers, MA) and the eluate was passed through 3K Microsep filters (Filtron, Northborough, MA) by centrifugation at 8,500 x *g* at 4°C.

Isolation of detergent solubilized molecules:

Large scale procedure:

The pellet following 100,000 x *g* centrifugation of 1 x 10^{11} amastigotes was disrupted using a glass rod, solubilized in PBS containing protease inhibitors and placed in polypropylene tubes. An equal volume of PBS containing detergent solution was added to the tubes and the suspension incubated on ice for 1 hr with intermittent vortexing. The suspension was placed in a fresh Beckman tube and centrifuged at 100,000 x *g* for 1 hr at 4°C. Following centrifugation, the supernatant containing the detergent solubilized proteins was collected and stored at -80°C.

Iodinated amastigotes:

Iodinated, lysed amastigotes were placed into 11x32 mm Quick-seal polyallomer bell-top ultracentrifuge tubes (Beckman) using a 3 c.c. syringe and 18 gauge needle, the tubes were heat sealed and centrifuged at 100,000 x *g* for 1 hr in a Beckman Tabletop TLA ultracentrifuge. A hole was pierced in the necks of the tubes using a 23 gauge needle and the supernatant was withdrawn from the tubes, and placed on ice. To gain access to the pellet, the necks of the tubes were cut using a scalpel. The pellet was homogenized using a glass rod, solubilized in PBS and placed in an eppendorf tube. An equal volume of PBS containing detergent solution was added to the tubes and the suspension incubated on ice

for 1 hr with intermittent vortexing. The suspension was placed in a fresh tube and centrifuged at 100,000 x *g* for 1 hr at 4°C. Following centrifugation, the supernatant containing the detergent solubilized proteins was collected.

Triton X-114 phase condensation:

The procedure of Bordier (1981) was followed for detergent solubilization of amastigote membranes. Briefly, intact amastigotes were solubilized on ice in 10 mM Tris-HCl pH 7.4, 1% Triton X-114 and protease inhibitors for 1 hr. The solution was layered onto 6% sucrose in Tris buffer, preheated to 30°C, and left for 3 min. at 30°C. The suspension was centrifuged for 10 min. at 300 x *g* at RT and the pellet at the bottom of the tube, containing detergent solubilized integral membrane molecules, was collected.

Acetone precipitation of proteins:

Proteins were concentrated using acetone precipitation. An equal volume of -20°C acetone (BDH) was slowly added to the sample with gentle mixing. The sample was left on ice for 15 min. then centrifuged at 13,000 x *g* for 5 min. The pellet was re-suspended in PBS.

Electron microscopy of amastigotes:

Amastigotes, purified from the footpads of *scid* mice using the technique for protein purification, were placed in a 15 ml polypropylene tube at a concentration of $5X10^8$ cells and centrifuged at 800 x *g* for 10 min. The cell pellet was re-suspended in 8 ml of 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 (cacodylic acid, sodium salt trihydrate; Baker Chem Co, Phillipsburg, NJ). After a 2 hr incubation at RT, the cells were washed twice in cacodylate buffer, at 800 x *g* for 10 min. Cells were re-suspended in 0.5 ml of 2% OsO_4 (J.B. EM Services, U.K.) in cacodylate buffer and left for 1 hr at RT. Cells were pelleted by centrifugation and the OsO_4 removed by pipette. The cells were re-suspended in 2% buffered agar (Difco, Detroit, MN) pre-heated to 50-60°C. After hardening, the agar cube containing amastigotes was cut into 0.5-1 mm cubes.

The cubes were fixed in an increasing series of ethanol for 15 min. each, then suspended in 3, 15 min. changes of propylene oxide (Fisher, Fairlawn, NJ). After a 2 hr incubation in 25% Epon : 75% propylene oxide, the cubes were incubated overnight in 50% Epon : 50% polypropylene. The cubes were embedded in Epon in Beem capsules and left in a 60°C oven for 48 hr to polymerize.

Ultrathin sections were made using an Ultracut E microtome (Reichart-Jung, Austria) and photographed using a Philips T400 TEM.

Chromatography:

Superose 12- gel filtration:

Superose 12- gel filtration was carried out at RT using the fast protein liquid chromatography (FPLC) system from LKB (Pharmacia, Bromma, Sweden). Samples were diluted in PBS and either filter sterilized with a 0.22 µm filter (Millipore) or spun at 13,000 x *g* for 10 min, before injecting 100 µl of sample on the column. PBS was used as the running buffer at a flow rate of 0.3 ml/min.

Protein A- affinity chromatography:

Affinity chromatography using Protein A-agarose beads (Sigma) was performed at 4°C. The serum was diluted 50% in 0.1M phosphate pH 8.0 and adjusted to pH 8.1 with 1M Tris HCl pH 9.0. The column bed was washed at a flow rate of 0.4 -0.5 ml/min. with 0.1M phosphate. Antibodies were eluted from the column using 0.1M Citrate at a pH of 3.5 to elute all the different subclasses of IgG. The eluate was collected in tubes containing 1M Tris HCl pH 8.5 to neutralize the pH of the solution.

CM affi-gel blue:

CM affigel blue (Bio-Rad) was carried out at 4°C on a 1.5x30 ml column with a 35 ml bed volume. Samples were run using a conventional chromatography system (Isco, Lincoln, NE) with 10 mM K_2HPO_4 pH 7.2 / 0.15 M NaCl / 0.02% NaN_3 as the running buffer at a flow rate of 0.33 ml/min.

Photography:

Gels and blots were photographed using Tech Pan film (Kodak) in a 35 mm camera. The prints were developed on F5 high gloss paper (Kodak).

Statistics:

The data was presented as the mean ± standard error mean (S.E.M.). A one and two-way analysis of variance (ANOVA) were employed to determine significance.

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Chapter III: Response of *scid* Mice to Establishment of *Leishmania major* Infection. [1]

INTRODUCTION

In susceptible strains of mice such as the BALB/c, *L. major* replicates in the tissues and causes cutaneous and systemic disease that ultimately leads to death of the host. In contrast, resistant strains of mice (C57BL/6, C3H/HeN) resolve the infection and are protected against re-infection (Mitchell, 1983). A model that has been recently used to study infectious diseases is the severe combined immunodeficient mice (*scid*) which are defective in T- and B-cell functions (Bosma *et al.*, 1983). Infection of *scid* mice with *Leishmania* has not as yet been properly characterized. In preliminary studies I found that these mice were very susceptible to infection, producing greater numbers of amastigotes than the BALB/c mice. In addition, amastigotes from *scid* mice had fewer host components bound to their surface. These two findings revealed that *scid* mice would be an excellent source of amastigotes. Since I used *scid* mice as a source of parasite, the course of infection in these mice needed to be characterized.

It is established that resistance to *L. major* is dependent upon the presence of lymphokines elaborated by helper T cells (Th) and competent effector cells, the macrophages. Removal of interferon gamma (IFNγ) using anti-cytokine antibodies renders the normally resistant mouse (C3H/HeN) susceptible to infection (Belosevic *et al.*, 1989; Sadick *et al.*, 1990; Scott, 1991). The susceptibility to *L. major* is dose-dependent because BALB/c mice also resolve the infection, when inoculated with low or sub-optimal numbers of parasites (Belosevic and Guy, 1992; Bretscher *et al.*, 1992). However, mice inoculated with a sub-optimal number of parasites and treated with anti-IFNγ antibody become susceptible to the infection (Belosevic and Guy, 1992). To successfully alter the course of infection in these mice, anti-IFNγ antibody must be administered prior to or during the first two to three days after inoculation of the parasites. Resistance is not affected when anti-IFNγ antibody is administered to mice four days after inoculation with *L. major*.

The requirement for IFNγ during the initiation of infection suggests that T cell-independent mechanisms may control the early clearance of

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[1] A version of this chapter has been published: Guy and Belosevic. Clin. Exp. Immunol. 1995. 100:440-5.

infection with *L. major*. The cellular source of IFNγ that facilitates the early clearance of the parasites from host tissues remains to be determined. A possible source of IFNγ at the inflammatory site is the natural killer (NK) cell (Handa *et al.*, 1983; Dorshkind *et al., 1985;* Perussia, 1991) IFNγ-producing NK-cells have been implicated in T-cell independent partial resistance to another intracellular pathogen, *Listeria monocytogenes* (Bancroft *et al.*, 1989; Wherry *et al.*, 1991). Although *scid* mice lack T and B-cells, they possess NK cells that produce IFNγ (Dorshkind *et al.*, 1985; Bancroft *et al.*, 1989; Wherry *et al.*, 1991).

I found that *scid* mice are susceptible to *L. major* and that they harbor high numbers of parasite in the footpads, and in later stages of infection, in the spleen and liver. However, there was no evidence of IFN$_\gamma$-dependent control of resistance to infection in *scid* mice. Instead, *scid* mice demonstrated a selective inhibition of inflammation at the site of inoculation during the initiation of *L. major* infection.

RESULTS

Course of infection:

The course of infection in the two strains of mice, *scid* and BALB/c, was compared when mice were given different numbers of parasites. *Scid* and BALB/c mice that received a high dose of amastigotes (1.5×10^6/mouse) were highly susceptible to infection, but the progression of the infection differed between mouse strains. A delay in lesion development occurred in *scid* mice during the first four weeks after inoculation, compared to BALB/c that received an equal dose of amastigotes (Figure 3-1). By week 5, lesion size was similar in both strains of mice. The number of parasites per footpad followed a similar trend. There was a significantly lower number of parasites per footpad in *scid* mice compared to BALB/c in the first two weeks of infection, but by week 3 after infection, the numbers of parasites in the footpads were similar between the strains. Four weeks after the inoculation, the number of parasites in the footpads of *scid* mice was significantly higher than that of BALB/c: $2.70 \pm 0.62 \times 10^8$ and $2.76 \pm 0.80 \times 10^7$ for *scid* and BALB/c mice, respectively (Figure 3-1).

I also estimated the number of parasites in the liver and spleen of mice 6 weeks after infection. *Scid* mice had significantly higher numbers of amastigotes in the two organs (Table 3-1). Although an occasional amastigote

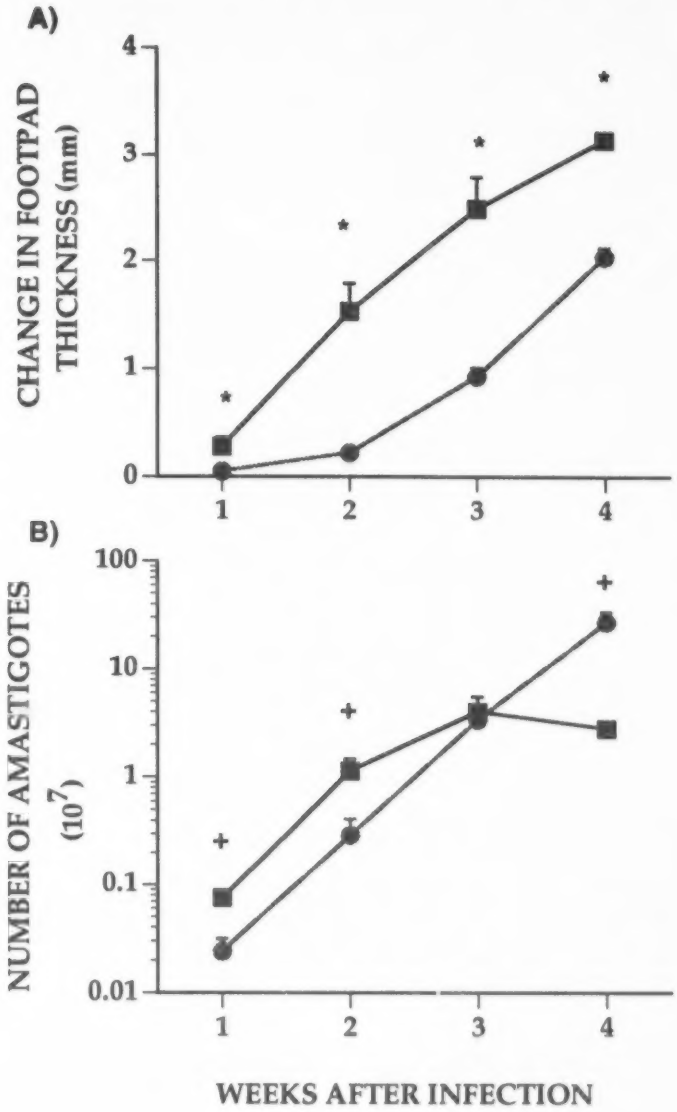

Figure 3-1: The course of infection in mice infected with *L. major*. BALB/c mice data are depicted by squares and *scid* mice data by circles. A) The change in footpad thickness of each mouse was measured by caliper and the size of the lesion determined by subtracting the depth of the uninfected contralateral footpad. The data are pooled from 3 separate experiments and are presented as the mean ± S.E.M. BALB/c mice, n=13 and *scid* mice n=35. (*) significantly different $p<0.0001$. B) The parasite number was estimated using a limiting dilution assay. Each point represents the mean ± the S.E.M. of 5 mice per group. The data are representative of 2 separate experiments. (+) significantly different $p<0.05$.

Table 3-1: The establishment of infection with *L. major* in the footpad, liver and spleen of *scid* and BALB/c mice.

MOUSE	FOOTPAD		LIVER		SPLEEN	
	Amastigotes[a] ($x10^7$)	Size (mm)	Amastigotes[b] ($x10^7$)	Weight (gm)	Amastigotes[a] ($x10^7$)	Weight (gm)
scid	105.8 ± 17.95 *	3.58 ± 0.78	17.5 ± 9.12 *	1.39 ± 0.05	1.51 ± 0.45 *	0.14 ± 0.01
BALB/c	6.6 ±1.46	3.28 ± 0.41	<1x 10^3	1.20 ± 0.03	<1 x 10^3	0.28 ± 0.01

Mice were inoculated in the left footpad with $1.5x10^6$ amastigotes. Disrupted tissues of mice were examined for the presence of parasites 6 weeks after infection. The data represent the mean ± SEM of 5 mice per group. The data are representative of 2 experiments with similar results. a: Total number of amastigotes per organ, b: Number of amastigotes per gm of liver. (*) Significantly different ($p<0.01$) compared to BALB/c mice.

could be detected in tissues of BALB/c mice, I was unable to accurately quantify their numbers due to the detection limit of the procedures (< 1 X 10^3 organisms).

Treatment of mice with anti-IFNγ antibody:

Although high numbers of amastigotes were observed in the footpads of *scid* mice, and early metastasis of the parasites to the spleen and liver, there was a delay in lesion development and amastigote production in the first two to three weeks after inoculation with *L. major*. To determine whether IFNγ participated in host defense during the initiation of *L. major* infection, *scid* mice were treated with anti-IFNγ antibody at the time of inoculation with 1.5 x 10^6 amastigotes. Antibody treatment did not alter the course of infection in *scid* mice, whereas infection was exacerbated in the susceptible BALB/c mice (Figure 3-2 A.). There were no differences in either lesion development or in the number of amastigotes per footpad in treated *scid* mice.

In a previous report, we showed that parasites fail to establish in BALB/c mice inoculated with a sub-optimal dose of promastigotes (Belosevic and Guy, 1992). Treatment of these mice with anti-IFNγ at the time of inoculation with the parasites, results in the establishment of *L. major*. However, a 4-day delay in administration of anti-IFNγ abrogates this effect (Belosevic and Guy, 1992). In contrast to BALB/c mice, *scid* mice given a low dose of parasites (1 x 10^2 amastigotes or 1 x 10^3 PNA^- promastigotes) became infected (Figure 3-2 B.). A measurable change in lesion size did not occur until approximately week 5 after infection, but by week 9, mice had very large lesions and the average number of amastigotes per footpad was 1.94 ± 0.56 x 10^9. High numbers of amastigotes were also detected at week 9 in the spleen and liver of these mice: 9.6 ± 4.1 x 10^7 and 3.4 ± 0.1 x 10^6, respectively. Interestingly, administration of anti-IFNγ did not alter the course of the infection in *scid* mice that received a sub-optimal dose of parasites. A similar course of infection and lack of response to anti-IFNγ treatment was obtained whether mice were infected with the low dose of either 1 x 10^2 amastigotes or 1 x 10^3 PNA^- promastigotes.

Intracellular killing and NO_2- production:

To further examine the role of IFNγ a comparison was made of the ability of peritoneal macrophages to kill the intracellular amastigotes in vitro.

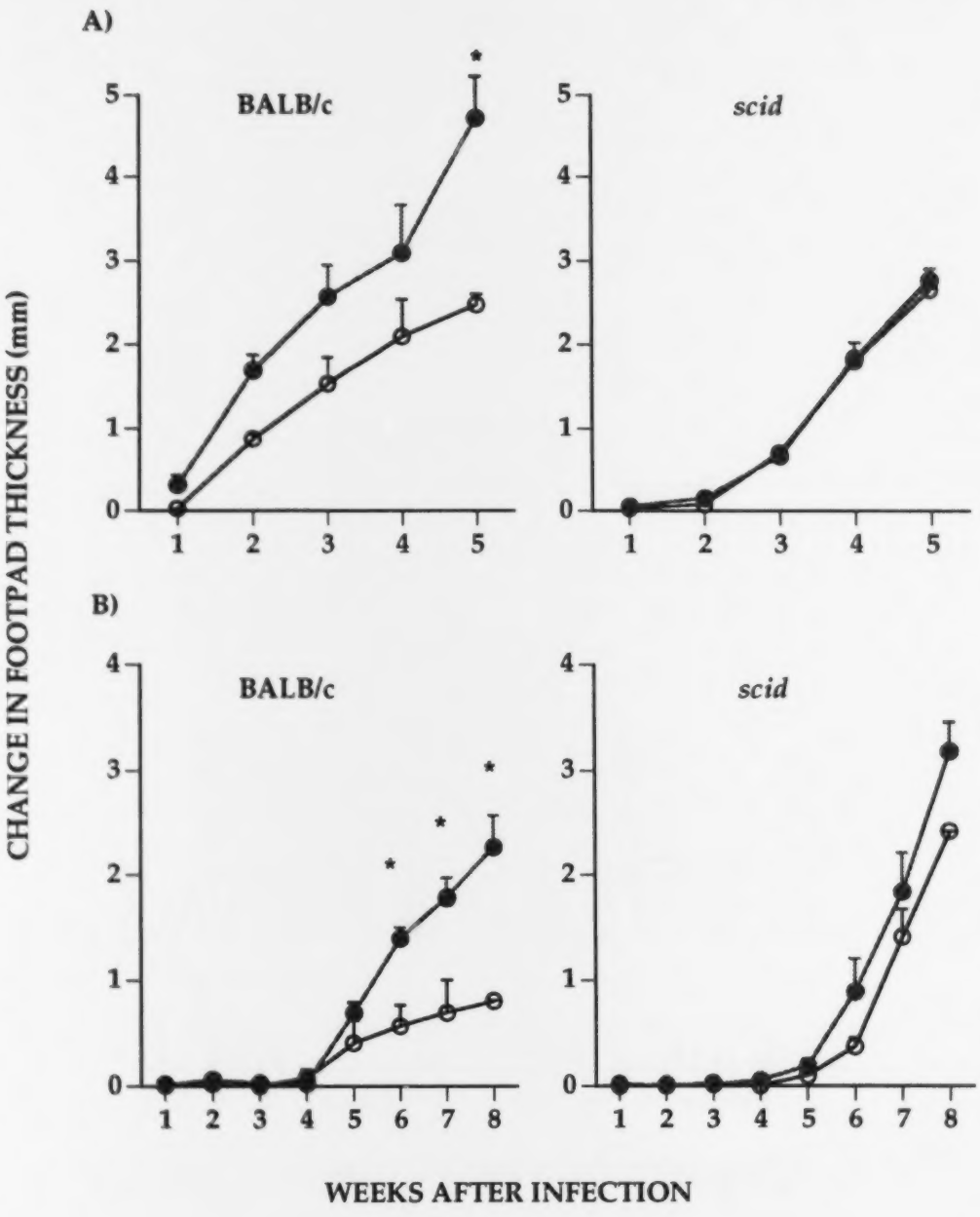

Figure 3-2: The course of infection in mice infected with *L. major* and treated with anti-IFNγ antibody. BALB/c and *scid* mice were infected with either a high dose A) or a low dose B) of *L. major*. Mice were treated with either anti-IFNγ (DB-1) (closed circle) or an irrelevant antibody (open circle). The change in footpad thickness was measured by caliper and the size of the lesion determined by subtracting the depth of the uninfected contralateral footpad. Each point represents the mean ± S.E.M. of 5 mice per group. The data are representative of 2 experiments with similar results. (*) significantly different ($p<0.05$) from treatment with irrelevant mAb.

Only macrophages from *Leishmania* -resistant C57BL/6 mice were capable of killing amastigotes; maximal killing was observed when 50 U/ml of rIFNγ was added to the cultures (Figure 3-3). There was no reduction in the percent of infected macrophages from either the *scid* mice or BALB/c mice after the in vitro treatment of macrophages with up to 200 U/ml of rIFNγ. However, a small reduction did occur in the number of amastigotes per macrophage in cultures treated with rIFNγ. A reduction of 17%, 25% and 60% for *scid*, BALB/c and C57BL/6 macrophages, respectively, was observed in the presence of 50 U/ml of rIFNγ.

Macrophages from all three mouse strains produced NO_2^- in response to rIFNγ. The greatest amount of NO_2- was seen in macrophages from C57BL/6 mice followed by BALB/c, while *scid* macrophages produced the lowest amounts (Figure 3-4 A). The same pattern of NO_2- production was observed when cells were stimulated with LPS (10 ng/ml) and increasing concentrations of rIFNγ (Figure 3-4 B). The statistical analysis is presented in Table 3-2. These data further support the observations that IFNγ does not regulate the initiation of *L. major* in this immunodeficient host.

Inflammatory response in *scid* mice:

Since the delay in development of the footpad lesions in *scid* mice did not appear to be under IFNγ control, I postulated that the reduced lesion size may be due to the failure of these mice to mount a timely inflammatory response at the site of infection. To test this hypothesis, I estimated the number of nucleated cells in the footpads, and popliteal and inguinal lymph nodes of infected mice at weekly intervals after the inoculation of parasites. Significantly fewer cells in the footpads of *scid* compared to BALB/c mice, were observed at week 2 and 3 after inoculation (Table 3-3). By week 4; however, the numbers of nucleated cells in the two strains of mice were similar: $2.11 \pm 0.37 \times 10^7$ and $6.50 \pm 2.3 \times 10^7$, in *scid* and BALB/c mice, respectively.

A significant increase was observed in the number of cells in popliteal lymph nodes of infected compared to non-infected mice. By week 1 the number of cells in infected BALB/c mice was significantly greater than in non-infected BALB/c mice. In contrast, a significant increase in the number of cells in infected *scid* mice, compared to non-infected mice, did not occur until week 2 of infection. In addition, BALB/c mice had significantly greater

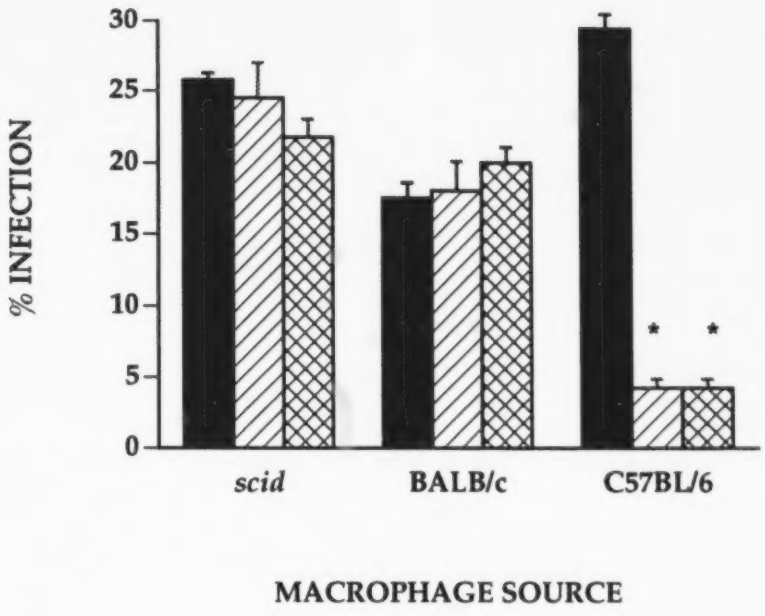

Figure 3-3: The percentage of IFNγ-treated macrophages infected with amastigotes of *L. major*. The concentration of IFNγ was either 0 units/ml (solid bars), 25 units/ml (vertical slashed bars) or 50 units/ml (hatched bars). The data are presented as the mean ± S.E.M. of 3 replicates per treatment and are representative of 4 separate experiments. (*) significantly different ($p<0.001$) from 0 units/ml IFNγ.

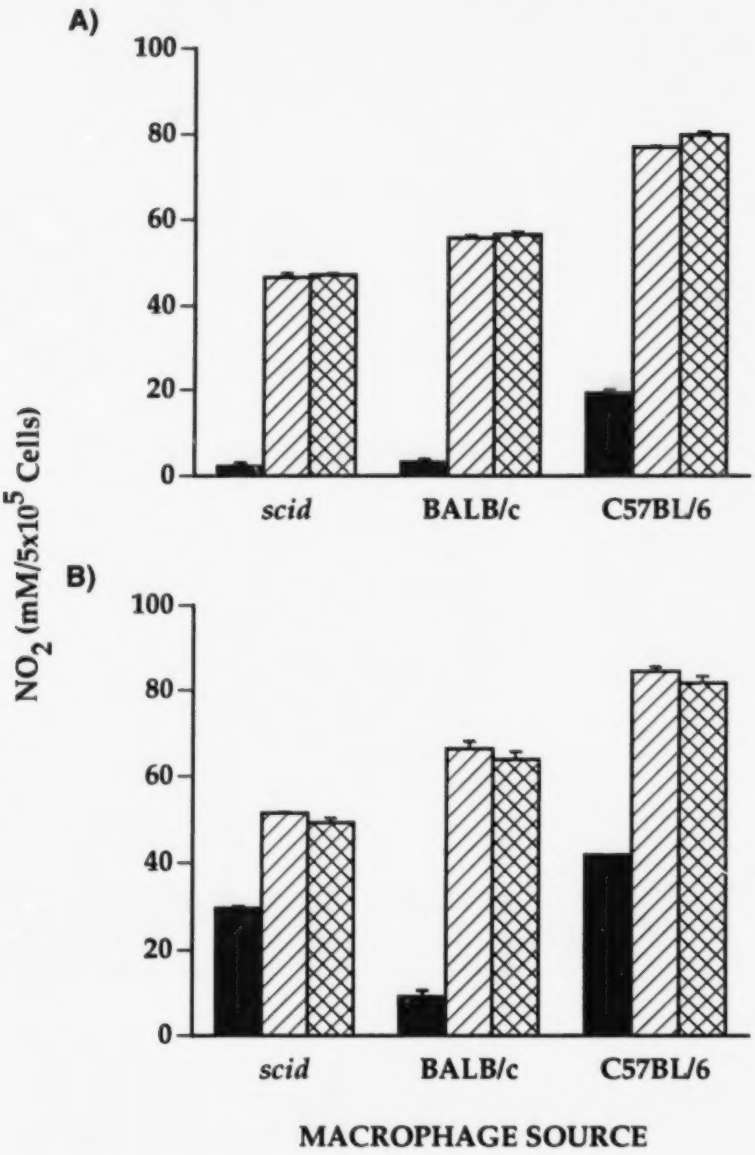

Figure 3-4: Nitric oxide response of macrophages after exposure to IFNγ. The concentration of IFNγ was either 0 units/ml (solid bars), 25 units/ml (vertical slashed bars) or 50 units/ml (hatched bars). A) The macrophages were infected at a 1:1 ratio of parasite to macrophage. B) The macrophages were stimulated with LPS (10 ng/ml). The data is represented as the mean ± S.E.M. of 3 replicates per treatment and is representative of 4 separate experiments. See Table 3-2 for statistical analysis.

Table 3-2: A three-way analysis of variance for comparing the nitric oxide response of macrophages after exposure to IFNγ and *L.major* or LPS.

SOURCE	df	Sum of Squares	F-value	P-value
Macrophage	2	6785	506	0.001
IFNγ	2	25016	1867	0.001
LPS/*L. major*	1	1441	215	0.001
Macrophage *IFNγ	4	1092	41	0.001
Macrophage *LPS/*L. major*	2	30	2	0.118
IFNγ *LPS/*L. major*	2	517	39	0.001
Macrophage *IFNγ *LPS/*L. major*	4	391	15	0.001
Residual	36	241	7	

P values from a means comparison test comparing the nitric oxide response of the three sources of macrophages stimulated with IFNγ.
df =1 in all cases.

Comparison	Sum of Squares	F-value	P-value
L. major infected macrophages:			
scid vs BALB/c	124	19	0.001
BALB/c vs C57BL/6	810	121	0.001
LPS stimulated macrophages:			
scid vs BALB/c	321	48	0.001
BALB/c vs C57BL/6	475	71	0.001

The data are presented for 50 units/ml of IFNγ because there was no difference in NO_2 response comparing 25 and 50 units.

Table 3-3: The number of nucleated cells in the footpads, popliteal lymph nodes, and inguinal lymph nodes of mice infected with *L. major*.

WEEK	NUMBER OF NUCLEATED CELLS ($x10^5$)					
	Footpad		Popliteal Lymph Node		Inguinal Lymph Node	
	scid	BALB/c	*scid*	BALB/c	*scid*	BALB/c
1	0.90 ± 0.09	1.08 ± 0.18	0.37 ± 0.30 *	45.53 ± 12.7	0.98 ± 0.49 *	22.64 ± 7.53
2	0.88 ± 0.36 *	19.00 ± 4.78	3.04 ± 0.59 *	185.18 ± 36.5	1.13 ± 0.56 *	37.30 ± 4.22
3	19.22 ± 2.82 *	220.60 ± 112.39	ND	ND	ND	ND

The number of nucleated cells in the organs of mice after infection with 1.5×10^6 amastigotes of L. major. The data is represented as the mean ± S.E.M. of 5 mice per group. and are representative of 2 experiments with similar results. * Significantly different from the other strain of mouse at that week of infection (ANOVA) $p<0.05$.

numbers of cells in popliteal lymph nodes week 1 and 2 after infection, than *scid* mice (Table 3-3).

The number of cells in the superficial inguinal lymph nodes did not significantly change during the infection in either *scid* or BALB/c mice. In addition, a comparison of the cells in the peritoneal cavity of non-infected and infected *scid* mice revealed no significant differences in the cell number of the different cell populations, except for an increase in the number of mast cells in infected *scid* mice, $5.40 \pm 0.12 \times 10^3$, compared to non-infected mice, $2.90 \pm 0.50 \times 10^3$ (Figure 3-5).

The inflammatory response of *scid* and BALB/c mice to *L. major* antigen or thioglycolate was compared, by enumerating the number of cells 4 days after injection of 1 mg of amastigote-derived antigen or 50 µl of 3 % thioglycolate into the left footpads. There was no increase in the numbers of cells in either strain of mice in the antigen injected left foot (Table 3-4), whereas a significant increase occurred in the thioglycolate stimulated foot (Table 3-5). Although the mice did not respond to *L. major* antigen in the footpad, examination of the popliteal lymph nodes revealed that BALB/c mice mounted a very strong response to the amastigote-derived antigens (Table 3-4). There was a 98-fold increase in the number of cells in the left node of mice that received antigen in the left foot. Whereas no change was observed in the popliteal lymph nodes of *scid* mice in response to injection of *L. major* antigen. The cell response to thioglycolate in the peritoneal cavity was similar in both strains of mice. A 17 and 22 fold increase in the number of macrophages following thioglycolate stimulation, was observed in *scid* and BALB/c mice respectively (Figure 3-6).

No difference was observed in the response to thioglycolate in mice that were infected with *L. major* 10 days prior to injection of 3% thioglycolate, compared to non-infected mice (Table 3-6). Therefore, infection with *L. major* does not impair the ability of the mice to mount an appropriate inflammatory response to thioglycolate.

DISCUSSION

The purpose of this study was to compare the host responses during an infection with *L. major* in normal and *scid* mice. I observed that in the first two to three weeks of infection the immunodeficient *scid* mice developed

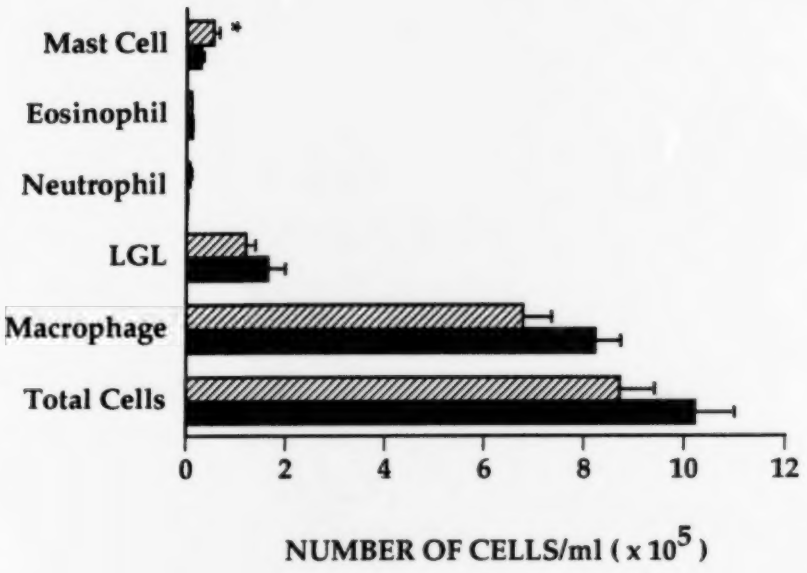

Figure 3-5: The number of resident peritoneal cells in non-infected (solid bars) and infected (slashed bars) *scid* mice. Infected mice were examined at 39 days post infection. The data is presented as the mean ± S.E.M. of 5 mice per group. (*) significantly different ($p<0.04$).

Table 3-4: The number of resident and inflammatory cells in the footpads and popliteal lymph nodes of *scid* and BALB/c mice following stimulation with *L. major* antigen.

MOUSE	FOOTPAD ($x10^4$)		POPLITEAL LYMPH NODE ($x10^4$)	
	Resident	Elicited	Resident	Elicited
scid	1.07 ± 0.26	0.84 ± 0.07	1.41 ± 1.29	0.51 ± 0.26
BALB/c	1.33 ± 0.35	1.71 ± 0.89	13.03 ± 12.92	1274.00 ± 107 *

Nucleated cells were determined in disrupted tissue four days after mice were injected with 50 µl of PBS into the right footpad and 50 µl of PBS containing 1 mg of whole, sonicated *L.major* antigen into the left footpad. Cells obtained from the right footpad or right popliteal lymph node are the resident cells and those from the left side of the body are elicited. The data represent the mean ± S.E.M. of 5 mice per group. (*) significantly different ($p<0.001$) from resident cells obtained from the same strain of mouse.

Table 3-5: The number of resident and inflammatory cells in the footpad of *scid* and BALB/c mice after stimulation with 3% thioglycolate.

MOUSE	NUMBER OF CELLS PER FOOTPAD ($x10^4$)	
	Resident	Inflammatory
scid	0.57 ± 0.09	10.01 ± 5.02
BALB/c	0.74 ± 0.10	28.77 ± 12.9

Nucleated cells were determined in disrupted tissue. Inflammatory cells were collected four days after 3% thioglycolate (0.05 ml/footpad) was injected into the mice. The data represent the mean ± S.E.M. of 4 mice per group for *scid* and 5 per group for BALB/c.

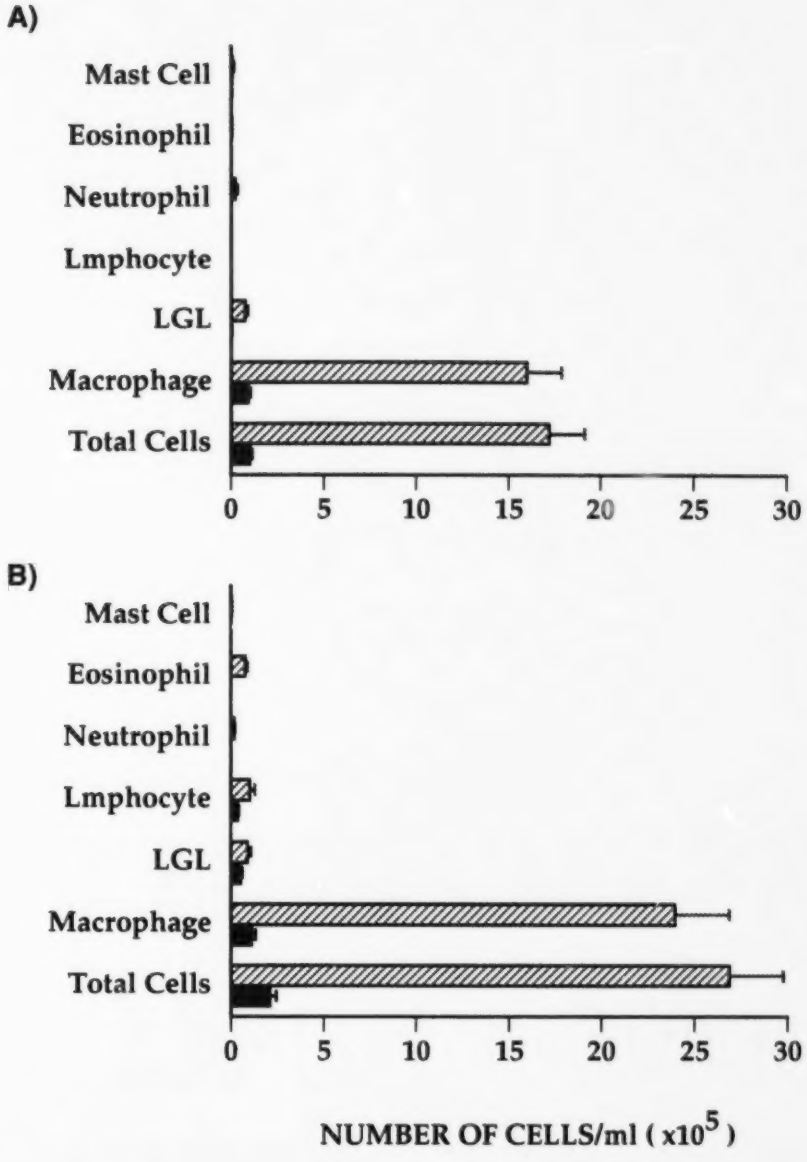

Figure 3-6: The number of resident and inflammatory cells in the peritoneal cavity of *scid* and BALB/c mice. A) *Scid* mice data. B) BALB/c mice data. The data is shown as the number of cells per ml of a 10 ml lavage of resident peritoneal (solid bars) and inflammatory cells (slashed bars). The data are presented as the mean ± S.E.M. of 4 mice per group.

Table 3-6: A comparison of the response of uninfected and infected *scid* and BALB/c mice after stimulation with 3% thioglycolate in the footpads.

MICE	TREATMENT	NUMBER OF NUCLEATED CELLS ($x10^4$) *scid*	BALB/c
Uninfected	Left Foot-PBS	0.30 ± 0.08	0.42 ± 0 .11
	Right Foot-3% Thioglycolate	47.97 ± 8.86	36.16 ± 7.72
Infected	Left Foot- PBS	12.25 ± 3.56	39.82 ± 7.42
	Right Foot-3% Thioglycolate	37.32 ± 7.06	33.16 ± 6.44

On day 0, mice were injected in the left footpad with either 50 µl of PBS or 50 µl containing $1.5x10^6$ amastigotes of *L. major*. At day 10 the mice were injected with 50 µl of 3% thioglycolate in the right footpad. Four days later (day14) the number of nucleated cells in each footpad were enumerated. The data are represented as the mean ± S.E.M. of 4 mice per group.

smaller footpad lesions compared to the susceptible BALB/c mice. In addition, the total number of parasites in the footpad lesions were significantly lower in *scid* mice, indicating that *scid* mice are more refractory to the establishment of *L. major* than BALB/c. In the later stages of the infection, as expected, *scid* mice were more susceptible to infection than BALB/c. A 10 to 100 fold increase in parasites was observed in the footpads of *scid* compared to the susceptible BALB/c mice. Replication of *Leishmania* amastigotes within macrophages initially occurs at the site of inoculation; however, by weeks 3 to 4 after infection, the parasites can be found in regional lymph nodes and eventually (6-8 weeks of infection) metastasize to the spleen, liver and bone marrow depending on the strain of *L. major* (Belosevic *et al.*, 1989). Hill (1986) reported very low numbers of *L. major* amastigotes in the spleen and liver of BALB/c mice as early as week 2 after infection, and by week 6 there were 1×10^5 and 5×10^4 parasites in the spleen and liver, respectively. We observed that the number of parasites in the spleen and liver of *scid* mice was 100 fold higher than that in BALB/c mice at week 6 after infection. This extreme susceptibility in the later stages of infection suggests that the amastigotes grow totally unchecked within the macrophages of *scid* mice.

Whether an animal is resistant or susceptible to *L. major* is determined very early in the course of infection. To render a resistant animal susceptible to *L. major*, anti-IFNγ must be administered within the first two days of infection, administration after day three does not alter the normal course of the infection (Belosevic and Guy, 1992; Leiby *et al.*, 1993). The source of IFNγ during the establishment of *L. major* infection is not known, but a likely candidate is the appropriately stimulated NK cell. NK cells produce IFNγ (Handa *et al.*, 1983), as well as other cytokines such as tumor necrosis factor-α (TNFα), granulocyte macrophage-colony stimulating factor (GM-CSF) and Interleukin-3 (IL-3) (for review see: Perussia, 1991).

To produce IFNγ, NK cells must be exposed to IL-12, also known as natural killer cell stimulating factor, a monocyte/macrophage and B cell product. Treatment of susceptible BALB/c mice with recombinant IL-12 in the first seven days of infection with *L. major* renders susceptible mice resistant to infection (Heinzel *et al.*, 1993; Sypek *et al.*, 1993) and cytokine patterns in the draining lymph nodes of these mice shift from a TH_2 to TH_1 profile. The IL-12 curative effect is dependent on IFNγ since co-treatment of

mice with IL-12 plus antibodies to IFNγ renders the mice susceptible to infection (Heinzel *et al.*, 1993). A role for NK cells in controlling the establishment of different pathogens has been shown for *Mycoplasma pulmonis* (Lai *et al.*, 1990) and *L. monocytogenes* (Bancroft *et al.*, 1989) and *Toxoplasma gondii* (Sher *et al.*, 1993). Although NK cells have been identified in lesion biopsies from *Leishmania*-infected humans (Ridel *et al.*, 1988), their presence at the lesion site within the first few days of infection has not been determined. Recently, Scharton and Scott (1993) demonstrated a role for NK cells in early resistance to *L. major* infection. NK cell depleted mice had greater numbers of parasites in the footpads 2 weeks after infection compared to normal mice.

Previous reports demonstrated that treatment of *scid* mice with anti-IFNγ exacerbates infection with *L. major* only when *scid* mice had received spleen cells from congenic BALB/c mice (Coffman *et al.*, 1991; Varkila *et al.*, 1993). Our study confirms the results that removal of IFNγ had no effect on the course of infection in *scid* mice given a high dose of *L. major*. We previously showed that the susceptible BALB/c mice control the establishment of sub-optimal inoculum of *L. major*, but that this control is abrogated by treatment with anti-IFNγ (Belosevic and Guy, 1992). Unlike BALB/c mice; however, *scid* mice were susceptible to low doses of *L. major* and the removal of IFNγ in vivo, using anti-IFNγ antibodies, did not alter the outcome of the infection. In addition, the stage of *L. major* used to infect the mice made no difference, as removal of IFNγ did not alter infection whether the mice were infected with a low dose of amastigotes or stationary phase promastigotes.

In this study, macrophages from *scid* mice were unable to kill *L. major* in vitro. Similar results were obtained using macrophages from BALB/c mice. In contrast, macrophages from the resistant C57BL/6 were very efficient in killing the intracellular amastigotes. Nacy *et al.* (1983) reported that BALB/c mice have a defect in lymphokine-induced macrophage intracellular destruction of *L. major*. They observed a very low killing by macrophages from BALB/c mice infected with *Leishmania*. The killing only occurred when high doses of lymphokine were used to activate the macrophages. I did not observe a reduction in the percentage of infected macrophages from BALB/c mice upon lymphokine stimulation. However, I tested the response of macrophages to IFNγ alone, whereas Nacy *et al.* (1983) used a lymphokine

supernatant obtained from the spleens of mice immunized intradermally with viable *Mycobacterium bovis*. Therefore, the low level macrophage killing of *L. major* reported in their study was probably due to a combined effect of different lymphokines.

Liew *et al.* (1991) reported the same defect in BALB/c peritoneal macrophages elicited by hydrolyzed starch. In their study, BALB/c macrophage killing of *L. major* in the presence of IFNγ and LPS was much lower than that of macrophages from the resistant CBA mice. These differences in killing correlated with lower levels of NO_2- production by macrophages from BALB/c mice. Recently, Stenger *et al.* (1994) demonstrated that susceptible BALB/c mice infected with *L. major* have lower levels of inducible nitric oxide synthase (iNOS) in the leishmanial lesion compared to that of the resistant C57BL/6 mice. The present data suggest that BALB/c congenic *scid* mice also have a macrophage defect in activation by IFNγ to kill intracellular *Leishmania*. *Scid* macrophages were capable of producing NO_2- in response to *L. major* or LPS but at lower levels than macrophages from BALB/c mice. BALB/c macrophages in turn produced lower levels of NO_2- when compared to Leishmania-resistant C57BL/6 mice. Killing of *Listeria monocytogenes* by *scid* macrophages has been reported (Beckerman *et al.*, 1993); however, this occurred when both IFNγ and TNFα were added to cultures. The activation of *scid* macrophages by IFNγ alone was not reported. A single study demonstrated the production of NO_2^- by resident peritoneal macrophages from *scid* mice (Beckerman *et al.*, 1993).

The transient resistance to infection in *scid* mice during the initiation of *L. major* infection is followed by the extreme susceptibility to the parasite in the later stages of the infection. The extreme susceptibility to *L. major* in immunocompetant mice has been linked to a dominant TH_2 response in later stages of the infection. It is unlikely; however, that *scid* mice exhibited a TH_2-like profile of cytokine production. Although mast cells are present in *scid* mice, and they are known to produce IL-4 (Gordon *et al.*, 1990), they do so following cross-linking of their Fc receptors. Since *scid* mice lack antibodies, this potential source of IL-4 is unlikely. Therefore, increased susceptibility and disease progression in the *scid* mouse is not associated and/or caused by a dominant TH_2 response during the late stages of the infection. It appears that the mechanisms of control of *L. major* infection are complex, depending on a

multitude of factors and these factors may differ in hosts of different genetic backgrounds.

Lower susceptibility of *scid* mice to *L. major* during the initiation of infection may be explained by postulating that the development of the footpad lesion is, in part, regulated by the host's inflammatory response at the site of the infection. A decrease in the number of inflammatory macrophages at the lesion site could slow down the multiplication of the parasites in the footpads. The *scid* mice were able to respond to a non-specific irritant, thioglycolate, that does not require T cell involvement to induce a rapid accumulation of macrophages at the site. However, a number of molecules, called chemokines, that induce macrophage recruitment are either T cell products or macrophage products that are dependent on stimulation by lymphokines for their secretion. For example, IL-4 a Th2 cell product, is chemotactic for macrophages in vitro (Hiester *et al.*, 1992), as is TGF-β a T and B cell product (Wahl *et al.*, 1987). IFNγ-stimulated macrophages produce the chemokine IP-10 (Kaplan *et al.*, 1987). Thus, T cell involvement may be important in initial recruitment of macrophages to rapidly establish *L. major* infection. In the later stages of infection the *scid* mice had very large numbers of macrophages containing amastigotes in the lesion site, suggesting that recruitment is normal once the infection is well established.

The host's inflammatory response at the footpad level may have been inhibited by the parasite itself. We did not observe a significant increase in the number of cells in the footpad in response to 1 mg of *L. major* antigen, whereas 3% thioglycolate elicited over 18 fold increase in the number of cells in the footpads. It should be noted; however, that the infection with *L. major* did not alter the ability of the mice to respond to thioglycolate. Previous studies have shown that *scid* mice respond differently to different antigens. For example, macrophage Ia expression was elicited by *L. monocytogenes*, *Salmonella typhimurium*, Bacillus Calmette-Guerin (BCG) and *Corynebacterium parvum*, but not by key hole limpet hemocyanin (Wherry *et al.*, 1991; Bancroft *et al.*, 1986). Kaye and Bancroft (1992) demonstrated that spleen cells from *scid* mice produce TNFα and IFNγ in response to heat killed *L. monocytogenes*, but not in response to either the promastigote or amastigote stage of *L. donovani*. Pre-incubation of spleen cells with amastigotes of *L. donovani* inhibited IFNγ production by heat killed *L. monocytogenes* stimulated cells, suggesting that in addition to defective

processing of *Leishmania* antigens, the parasite itself may actively inhibit cell responsiveness (Kaye *et al.*, 1992). Similarly, Reiner *et al.* (1994) demonstrated that the promastigote stage of *L. major* did not induce cytokine responses from macrophages in the first week after infection, whereas the amastigote stage stimulated cytokine production. Whatever the mechanism, the recruitment of appropriate macrophages to the lesion site is necessary for the multiplication of the parasite and progression of the disease.

The finding that *scid* mice are extremely susceptible to *L. major* infection and produce 10-fold greater number of amastigotes than BALB/c mice was important for the present study. It provided a larger source of amastigotes to carry out the research and an alternative source of biologically relevant parasites. The next chapter describes the results of experiments designed to characterize the infection of macrophages in vitro with different sources of the parasite: promastigotes and amastigotes obtained form BALB/c and *scid* mice. Additionally, a comparison was made of the ability of macrophages obtained from different sources to phagocytose the parasite.

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Chapter IV: Comparison of the Susceptibility of Different Macrophage Populations to *L. major* In Vitro.

INTRODUCTION

Macrophages are very heterogeneous cells and can be differentiated by a number of characteristics which include morphology, intracellular enzymes, secreted products and cell surface receptors (for reviews see: Leenen and Campbell, 1993; Rutherford *et al.*, 1993). In addition, macrophages exhibit functional heterogeneity; phagocytic ability, cytotoxicity, production of bioactive molecules and regulation of inflammatory and immune responses (Nathan, 1987; Rutherford *et al.*, 1993). Macrophages can be isolated from a number of sites in the body in sufficient numbers to conduct in vitro experiments. Moreover, there are several macrophage-like cell lines available which enable studies of cells arrested at different stages of differentiation.

Many different macrophage populations have been employed to study the interaction with pathogens. To be a suitable host cell for *Leishmania* the macrophage must first phagocytize the parasite and then allow the pathogen to multiply within the cell. The macrophages most frequently used to study the interaction with *Leishmania* are the murine resident peritoneal macrophages (Blackwell *et al.*, 1985; Mosser and Edelson, 1985, Cooper *et al.*, 1988) and human monocyte-derived macrophages (Wilson and Pearson, 1988; Russell *et al.*, 1989; Da Silva *et al.*, 1989). Murine thioglycolate elicited macrophages (Handman and Spira, 1977; Chakraborty and Das, 1988), bone marrow derived-macrophages (Guy and Belosevic, 1993; Kelleher *et al.*, 1995), the macrophage cell line J774A.1 (Russell and Wilhelm, 1986; Kelleher *et al.*, 1995) and dermal macrophages and Langerhan cells (Locksley *et al.*, 1988) have also been used.

Very little comparative assessment of the ability of different macrophage populations to phagocytize a single species of *Leishmania* have been done. A study using amastigotes of *L. donovani* showed that highly phagocytic cells were from the bone marrow, spleen and the peritoneal cavity (Crocker *et al.*, 1987). Pappas (1983) compared the phagocytosis of *L. major* amastigotes by resident versus inflammatory peritoneal macrophages. The inflammatory cells were elicited using five different agents: thioglycolate, colloidal starch, latex beads, FBS or PBS. There were no differences in the

percent of macrophages infected with amastigotes after a 1 hr interaction (Pappas, 1983). In another study, the uptake of promastigotes of *L. major* into dermal macrophages was high, whereas no promastigotes entered epidermal Langerhan cells (Locksley *et al.*, 1988). Blank *et al.* (1993) also reported that Langerhan cells are less susceptible to *Leishmania*.

In this chapter, results of experiments designed to elucidate the basic parameters of entry of amastigotes into macrophages are presented. The purpose of these studies was to establish the basis for future experiments on the interaction of the parasite with macrophages in vitro. The experiments described in the second part of the chapter help determine whether macrophages obtained from mice, that are susceptible or resistant to *L. major* in vivo, have different phagocytic abilities in vitro. The results in the third part of the chapter deals with a comparison of the infection in different macrophage populations.

RESULTS

Entry of *Leishmania* into bone marrow-derived macrophages in vitro:

The majority of in vitro experiments performed in this thesis, were conducted using bone marrow-derived macrophages (BMDM). The BMDM were derived by stimulation of bone marrow cells with the growth factor, colony stimulating factor-1 (CSF-1) and were routinely used after 9-11 days of culture.

I examined the capacity of BMDM obtained at different days of cultivation to ingest the parasite. BMDM obtained after culture for 3 days in the presence of CSF-1 phagocytized amastigotes. No differences were observed in the percent of infected cells comparing BMDMs cultured for 3 and 9 days: 57 ± 1.7% and 54 ± 0.6 %, respectively. However, examination of Giemsa stained cells revealed that only 60% of the cells in the day 3 culture were macrophages compared to 77% on day 6, and 96%, 97% and 99% on days 7 through 9. Phagocytosis of the parasites was not affected by either the removal of CSF-1 24 hr prior to infection of BMDM with parasites, just prior to infection, or in the continued presence of CSF-1 during phagocytosis. The addition of 10% fetal bovine serum (FBS) to the phagocytosis assay medium was not an absolute requirement. The percent of BMDM infected with promastigotes was 45 ± 2.0% and 36 ± 4.6 % in the absence and presence of

FBS, respectively. The percent of BMDM infected with amastigotes was 46 ± 0.8% and 50 ± 1.9 % in the absence or presence of FBS, respectively.

Since the isolation of amastigotes from the footpad took approximately 1 hr, experiments were performed to determine whether the isolation time altered the infectivity of the amastigotes. I observed that amastigotes were equally infective, immediately after isolation from the footpads or 2 and 4 hr after isolation: 63 ± 3.3, 63 ± 2.0% and 61 ± 0.3%, respectively.

The fact that BMDM were good hosts for *L. major* is supported by the data presented in Table 4-1. Promastigotes phagocytized by BMDM transformed into the amastigote stage and replicated.

Not surprisingly, the percent of infected macrophages was strongly influenced by the ratio of parasite to macrophage; increasing the number of parasites resulted in a greater percent infection (Figure 4-1). The uptake of amastigotes obtained from BALB/c mice was greater than that of amastigotes from *scid* mice or the promastigotes. To obtain a 50% infection in the BMDM required only a 2:1 ratio of BALB/c -source amastigotes as opposed to a 10:1 ratio of either *scid*-source amastigotes or promastigotes (Figure 4-1). Interestingly, the infectivity of amastigotes obtained from *scid* mice was similar to promastigotes; at a 2:1 ratio only 10-20% of BMDM became infected after 2 hr exposure to the macrophages. However, the entry of *scid*- and BALB/c-source amastigotes were equivalent after a 24 hr exposure. The maximum percentage of infected macrophages was approximately 80% using amastigotes at a 10:1 ratio (Figure 4-1), indicating the presence of a residual population of macrophages that are refractory to the parasite.

A comparison was also made of the ability of BMDM to phagocytize two different stains of *L. major*, the NIH 173 and NIH 80 strains. The BMDM were equally susceptible to BALB/c-source amastigotes of the two strains. The percent infected cells were 49 ± 1.3% for NIH 173 and 47 ± 1.8% for NIH 80. The same results were obtained with the promastigote stage: 46 ± 0.7% for NIH 173 and 45 ± 0.9% for NIH 80.

BMDM obtained from previously infected animals phagocytized the *scid*-source amastigotes to the same extent as BMDM obtained from non-infected animals (Table 4-2). However, a small but significant ($p<0.02$) increase was observed in the phagocytosis of the BALB/c-source amastigotes by BMDM obtained from infected *scid* mice compared to non-infected mice. The survival of amastigotes in the macrophages was similar. After 72 hr in

Table 4-1: The transformation of promastigotes into amastigotes within bone marrow-derived macrophages.

DAY IN CULTURE	% INFECTED MACROPHAGES	NUMBER OF PARASITES PER 100 MACROPHAGES
0	13 ± 1.7	100 ± 0
4	73 ± 0.3	685 ± 49
8	72 ± 2.3	731 ± 17
12	84 ± 1.5	750 ± 23
16	84 ± 2.0	1,000 ± 21

BMDM were infected with stationary phase promastigotes at a ratio of 20 parasites : 1 macrophage. On the day of infection, day 0, and every 4 days thereafter, cells were spun onto glass slides, stained and examined for the percent infection and number of amastigotes per macrophage. The data are presented as the mean ± S.E.M of three replicates examined at each time point.

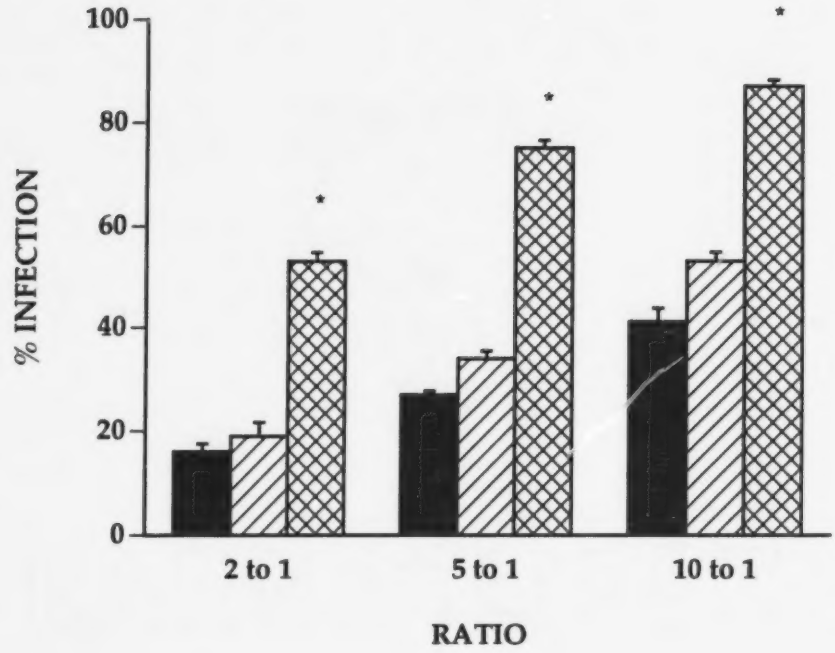

Figure 4-1: The percent infection of macrophages in vitro with *L. major* at different ratios of parasite to macrophage. The solid bars represent promastigotes, vertical slashed bars *scid*-source amastigotes and hatched bars BALB/c-source amastigotes. The BMDM wre infected for 2 hr. The data are presented as the mean ± S.E.M. Each group was performed in triplicate and the data are representative of at least 3 experiments with similar results. (*) Significantly different ($p<0.05$) than the other parasites at the same ratio.

Table 4-2: A comparison of the percent infection of macrophages, obtained from non-infected or *L. major* infected *scid* mice, with amastigotes.

MACROPHAGE SOURCE	AMASTIGOTE					
	BALB/c			*scid*		
	2:1	5:1	10:1	2:1	5:1	10:1
Non-infected	54 ± 0.7	78 ± 0.6	88 ± 0.9	34 ± 2.4	49 ± 1.5	64 ± 1.5
Infected	65 ± 0.9	87 ± 1.7	96 ± 2.1	33 ± 1.2	51 ± 1.7	59 ± 3.7

The data are presented as the mean ± S.E.M. of three replicates per group.

culture the percentages of infected cells were 86 ± 3.0% and 93 ± 1.8% for BMDM obtained from non-infected and infected mice, respectively. Thus, the infection of mice with *Leishmania* did not reduce the phagocytic ability of the BMDMs or their suitability as a host cell.

Comparison of macrophages obtained from mice, susceptible or resistant to *L. major* in vivo:

Interestingly, the strain of mouse used to obtain BMDM (*Leishmania* resistant or susceptible animals) did not alter the percent of macrophages that phagocytized the parasite or the number of parasites that were engulfed by individual BMDMs (Table 4-3).

I also compared the phagocytic ability of four macrophage cell lines that were established from cells obtained from mice that are susceptible or resistant to *Leishmania*. No statistically significant differences (Table 4-4) were observed when comparing the phagocytosis of either BALB/c-source or *scid*-source amastigotes by the four macrophage cell lines (Table 4-5).

Comparison of the susceptibility of different macrophage populations to different sources of *L. major*.

A comparison was made of the percent infection of macrophages after 2 hr exposure to the parasite to assess whether different macrophage populations had differential abilities to phagocytize *L. major*. There was very little variation within individual experiments, whereas the variation from experiment to experiment was high. For these reasons, the means of multiple experiments were used to calculate the ANOVA's.

At a 2:1 infection ratio, a two-way analysis of variance revealed a significant interaction between the amastigotes and the macrophages (Table 4-6). Linear contrast tests were used to determine where these differences occurred. The tests were adjusted using the Bonferroni method (Godfrey, 1985) and values of $p<0.05$ were considered significantly different.

There were no differences observed in the phagocytosis of BALB/c-source (Figure 4-2) or *scid*-source (Figure 4-3) amastigotes by the macrophage populations at a 2:1 ratio of parasite to macrophage. However, significantly more BALB/c-source amastigotes were phagocytized by BMDM than the *scid*-source amastigotes.

Table 4-3: The percent infection and survival of amastigotes in macrophages obtained from three strains of mice.

TIME (hours)	MACROPHAGE SOURCE	AMASTIGOTE SOURCE			
		BALB/c		*scid*	
		PERCENT INFECTION	NUMBER PER 100 MØ	PERCENT INFECTION	NUMBER PER 100 MØ
2	C57BL/6	38 ± 2.3	176 ± 4.5	35 ± 0.5	156 ± 1.0
	BALB/c	46 ± 2.5	197 ± 6.4	33 ± 1.2	153 ± 4.0
	scid	43 ± 0.9	207 ± 9.6	30 ± 3.2	147 ± 1.2
24	C57BL/6	50 ± 1.2	243 ± 2.5	50 ± 2.2	205± 3.4
	BALB/c	48 ± 1.2	228 ±11.1	45 ± 0.3	203 ± 1.7
	scid	47 ± 2.9	214 ± 8.1	45 ± 0.3	189 ± 4.8
72	C57BL/6	46 ± 0.7	242 ± 12.7	49 ± 4.9	232± 19.4
	BALB/c	49 ± 2.2	243 ± 10.5	49 ± 1.5	185 ± 1.3
	scid	58 ± 0.9	269 ± 18.1	54 ± 1.5	224 ± 5.0

The ratio of amastigote to macrophage was 2:1 for all strains of mice. MØ = Macrophage. The data are presented as the mean ± S.E.M. of 3 replicates per group and are representative of 2 experiments with similar results.

Table 4-4: A two-way analysis of variance for comparing the entry of BALB/c-source and *scid*-source amastigotes into different macrophage cell lines.

SOURCE	df	Sum of Squares	F-Value	P-Value
Amastigote	1	2.069	17.266	0.001
Macrophage	3	0.880	2.449	0.110
Amastigote * Macrophage	3	0.199	0.522	0.656
Residual	13	1.558	0.120	

The data were from the means of replicate experiments and were transformed using the formula arcsin(sqrt).
n= 2 for *scid*-source amastigotes; n= 3 for BALB/c-source amastigotes into P388D.1, ANA-1, BA-1; n=4 for BALB/c-source amastigotes into J774A.1 cells.

Table 4-5: The entry of amastigotes into macrophage cell lines derived from different strains of mice.

CELL LINE	AMASTIGOTE SOURCE	
	BALB/c	*scid*
	Percent Infection	Percent Infection
J774A.1	37 ± 10.2	11 ± 9.5
P388D.1	35 ± 2.0	14 ± 5.0
ANA-1	64 ± 4.6	30 ± 14.0
BA-1	38 ± 10.3	9 ± 4.5

The macrophages were exposed to amastigotes at a 2:1 ratio, for 2 hr. The data are presented as the mean ± S.E.M. of multiple experiments. n=2 for *scid*-source amastigotes in all cell lines; n=3 for BALB/c-source amastigotes in P388D.1, ANA-1 and BA-1; and n=4 for BALB/c-source amastigotes in J774A.1 cells.

Table 4-6: A two-way analysis of variance for comparing the entry of BALB/c-source and *scid*-source amastigotes into different macrophage populations.

SOURCE	df	Sum of Squares	F-Value	P-Value
Amastigote	1	0.318	9.357	0.007
Macrophage	2	0.070	1.026	0.377
Amastigote * Macrophage	2	0.327	4.809	0.020
Residual	19	0.645	0.034	

The data were from the means of replicate experiments and were transformed using the formula arcsin(sqrt).
n= 2 for *scid*-source amastigotes into rPC, iPC; n= 3 for BALB/c-source amastigotes into iPC; n=5 for entry of BALB/c-source amastigotes into rPC; n=6 for BALB/c-source amastigotes into BMDM; and n=7 for entry of *scid*-source amastigotes into BMDM.

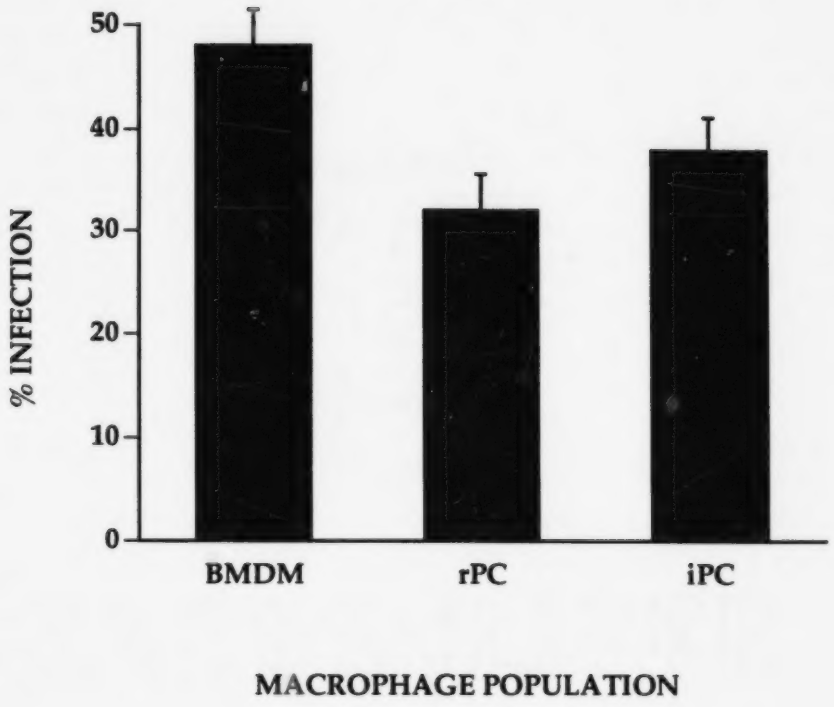

Figure 4-2: The percent infection of different macrophage populations with BALB/c-source amastigotes. The macrophages were exposed to the parasite for 2 hr at an infection ratio of 2:1. The data are presented as the mean ± S.E.M. of 6 experiments for BMDM, 5 for rPC and 3 for iPC.

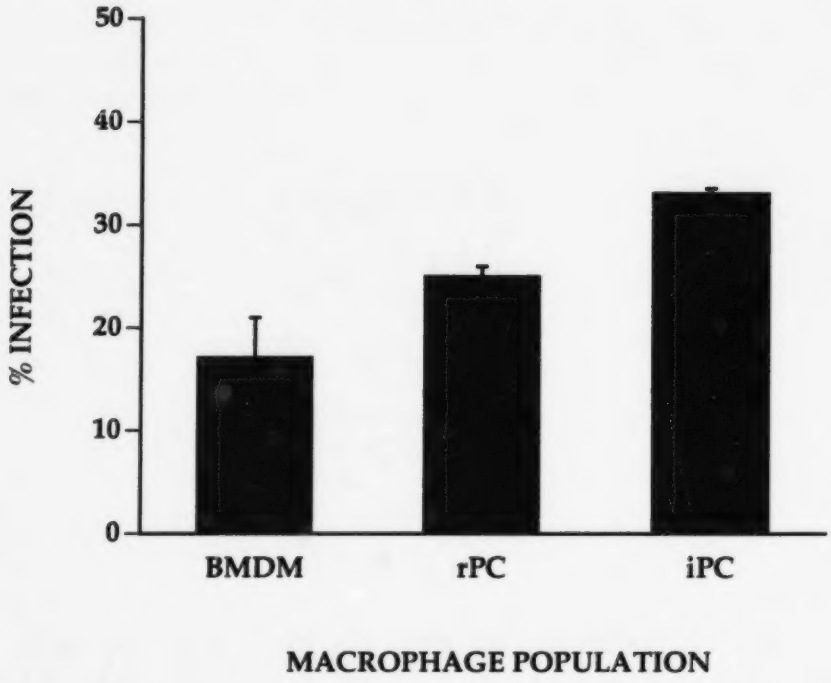

Figure 4-3: The percent infection, at a 2:1 ratio, of different macrophage populations with *scid*-source amastigotes. The macrophages were exposed to the parasite for 2 hr at an infection ratio of 2:1. The data are presented as the mean ± S.E.M. of 7 experiments for BMDM, 2 for rPC and 2 for iPC.

At a 10:1 infection ratio, a two-way analysis of variance revealed a significant interaction between the parasites and the macrophages (Table 4-7). There were no differences in the phagocytosis of *scid*-source amastigotes by the different macrophage populations (Figure 4-4). However, BMDM phagocytized significantly ($p<0.05$) more promastigotes than the rPC or the iPC (Figure 4-5). Comparing the phagocytosis of the promastigotes with the *scid*-source amastigotes revealed significantly more ($p<0.05$) of the *scid*-source amastigotes were phagocytized, by rPC and iPCs, than the promastigotes.

Two macrophage cell lines that express very little or no phagocytic receptors on their surface, were also studied. Infection of the two lines, the myeloblast cell line M1 and the immature macrophage line WEHI-3, was very low. At a ratio of 10:1 *scid*-source amastigote to macrophage, the mean ± S.E.M. percent infection was 2 ± 0.7% and 11 ± 6.9% for M1 and WEHI-3, respectively. There was no significant difference ($p<0.13$) in the percent of infected cells between the two macrophage cell lines. Similar results were obtained with the BALB/c-source amastigotes, but, no promastigotes were observed within either M1 or WEHI cells.

DISCUSSION

The objectives of the research described in this chapter were to elucidate the basic parameters of entry of amastigotes into macrophages, and to compare the ability of different macrophage populations to phagocytize *L. major* in vitro. The CSF-1 derived BMDM were chosen as the primary macrophage population for my studies because these macrophages are relatively homogenous regarding the surface antigen expression of Mac-1, and Mac-2 (Falk and Vogel, 1988; Walker *et al.*, 1985) morphology and function, and they readily phagocytize both stages of the parasite. BMDM are morphologically and functionally different when derived using CSF-1 or GM-CSF. By day 7 in culture CSF-1 derived BMDM are more homogenous in size, and are more phagocytic than GM-CSF derived BMDM, especially by the FcR (Falk and Vogel, 1988). Interestingly, Rutherford and Schook (1992) found that GM-CSF derived macrophages were more phagocytic for *Listeria monocytogenes* than CSF-1 derived macrophages. This observation was reversed; however, when the macrophages were activated with IFNγ and LPS. The two macrophage populations differ in tumoricidal abilities, GM-CSF-

Table 4-7: A two-way analysis of variance for comparing the entry of promastigotes and *scid*-source amastigotes into different macrophage populations.

SOURCE	df	Sum of Squares	F-Value	P-Value
Parasite	1	1.448	35.605	<0.001
Macrophage	2	0.836	10.277	0.001
Parasite * Macrophage	2	1.030	12.668	<0.001
Residual	18	0.732	0.041	

The data were determined from the means of replicate experiments and were transformed using the formula arcsin(sqrt).
n=2 for *scid*-source amastigotes into iPC; n=3 for *scid*-source amastigotes into rPC and for promastigotes into iPC; n=4 for promastigotes into BMDM and rPC; n=8 for *scid*-source amastigotes into BMDM.

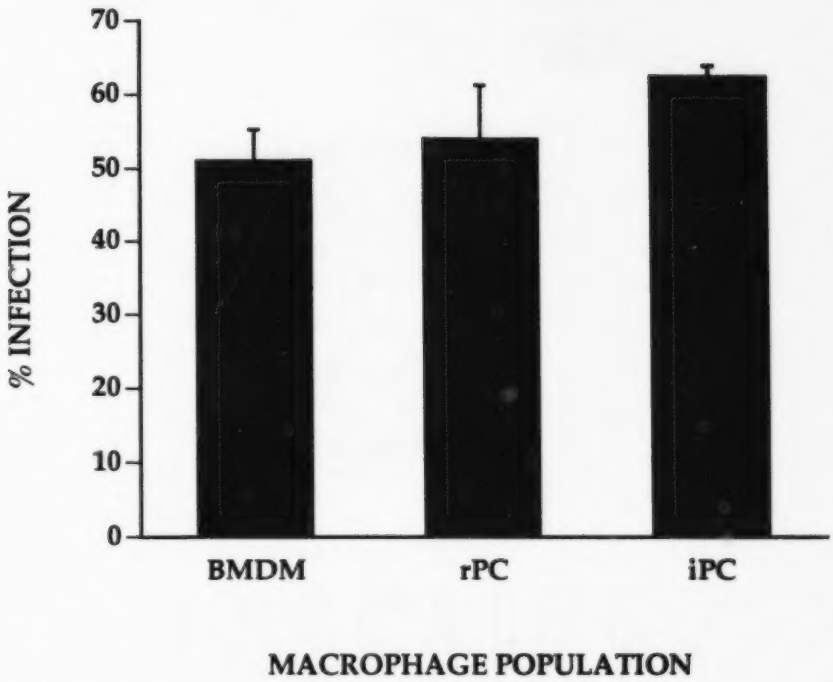

Figure 4-4: The percent infection at a 10:1 ratio, of different macrophage populations with *scid*-source amastigotes. The macrophages were exposed to the parasite for 2 hr at an infection ratio of 10:1. The data are presented as the mean ± S.E.M. of 8 experiments for BMDM, 3 rPC, and 2 experiments for iPC.

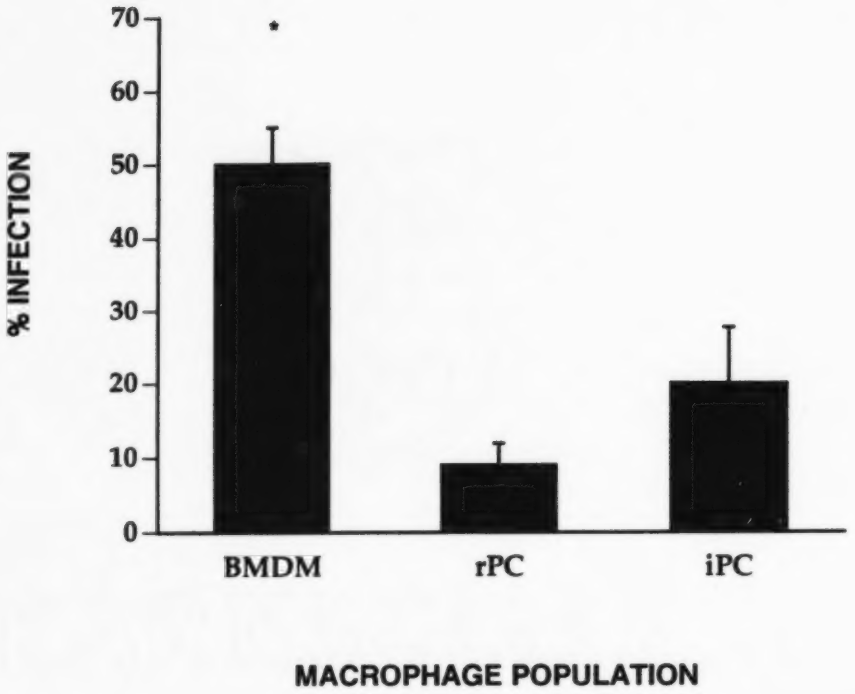

Figure 4-5: The percent infection of different macrophage populations with promastigotes of *L. major*. The macrophages were exposed to the parasite for 2 hr at an infection ratio of 10:1. The data are presented as the mean ± S.E.M. of 4 experiments for BMDM and rPC and 3 for iPC. (*) significantly different ($p<0.02$) from the other two populations.

derived BMDM are more tumoricidal than CSF-1 derived BMDM (Falk *et al.*, 1988a). Their responses to priming and triggering signals (Rutherford and Schook, 1992), secretory products, and ability to kill intracellular organisms (Rutherford and Schook, 1992) as well as Ia antigen expression also differ (Falk *et al.*, 1988b).

BMDM obtained after 3 and 9 days of culture with CSF-1 were equally phagocytic in this study. Leenen *et al.* (1994) demonstrated that BMDM elicited with CSF-1 express Mac-1 and Mac-2 on their surface by day 1-2. By day 3, close to 100% expression of these antigens occur; however, expression levels are lower than day 6 to 10 cultures (Leenen *et al.*, 1994). Day 4 BMDM cultures phagocytose Ig opsonized sheep red blood cells at a level similar to day 8 and 10 (Neumann and Sorg, 1980).

No differences were observed in the susceptibility of BMDM to two strains of *L. major*, the NIH 173 that is quick to metastasize to internal organs and the NIH 80 which is slow in metastasizing. This suggests that metastasis of *L. major* may not be dependent on the type of macrophage the parasite enters but that it is controlled by other mechanisms.

A previous infection with *L. major* did not affect the ability of BMDM derived from the exposed mice to become infected with the parasite in vitro or for the parasite to survive for 72 hr in these cells. Handman and Spira (1977) showed an inhibition of growth of amastigotes in macrophages in vitro when the macrophages were obtained from immune mice. However, these authors did not examine the uptake of the parasite into the cells in the first few hours after infection which may have influenced their findings.

The strain of mouse, whether resistant or susceptible to *L. major*, had no effect on the ability of the BMDM to phagocytose amastigotes in vitro or on survival of amastigotes. Furthermore, phagocytosis was similar by macrophage cell lines derived from susceptible and resistant mice. These results confirm a previous study using *L. major* in peritoneal macrophages (Nacy *et al.*, 1983) and further demonstrates that this occurs with two different sources of amastigotes. In a study using *L. donovani* promastigotes it was reported that liver macrophages obtained from resistant mice were less phagocytic than macrophages obtained from a susceptible strain of mouse. These differences did not occur in lung, spleen or peritoneal macrophages (Olivier and Tanner, 1987). On the other hand, Crocker *et al.* (1987) found no differences in the phagocytosis of *L. donovani* amastigotes after 1 to 2 hr

incubation of macrophages obtained from susceptible and resistant mice. However, they did observe that replication of amastigotes in liver macrophages was significantly greater in cells obtained from susceptible compared with resistant animals.

I did observe differences in the ability of different macrophage populations to phagocytose the parasite. This was dependent on which source of parasite was being studied. Differences between macrophage populations have been reported for the uptake of *L. donovani* amastigotes, whereby the most phagocytic cells were obtained from bone marrow, spleen and the peritoneal cavity. Lung and liver macrophages had low parasite loads (Crocker *et al.*, 1987). A previous study has demonstrated differential phagocytosis of promastigotes and BALB/c-source amastigotes of *L. major* into epidermal Langerhan cells (Blank *et al.*, 1993). The promastigote stage was not phagocytosed by these macrophages, whereas 20% of cells phagocytosed amastigotes at a 2:1 infection ratio. Although Langerhan cells phagocytose amastigotes they are not as susceptible compared to the macrophages used in this study. Interestingly, the amastigote stage does not replicate in Langerhan cells (Blank *et al.*, 1993). The authors hypothesized that these cells are important as antigen processing cells to initiate specific immune responses.

The majority of studies on the interaction of *L. major* promastigotes with macrophages have used human monocyte-derived macrophages, whereas studies on *L. donovani* were carried out using resident peritoneal cells. This must be due to the low percent of *L. major* promastigotes that are phagocytosed by peritoneal cells as seen in this study, whereas promastigotes of *L. donovani* are readily phagocytosed by these cells (Blackwell *et al.*, 1985). Resident peritoneal macrophages are also suitable hosts for other pathogens for example, unopsonized *Mycobacterium tuberculosis* is bound more efficiently by resident peritoneal macrophages than BMDM or J774A.1 cells. Thioglycolate and BCG elicited macrophages bound the *M. tuberculosis* very poorly (Stokes *et al.*, 1993). Therefore, not only are there differences in phagocytosis between the stages of the parasite but differences exist between species. This study demonstrated that BMDM are a good macrophage population to study phagocytosis of promastigotes of *L. major*.

The differences in susceptibility to infection of various macrophage populations are most likely due to differential expression of appropriate

receptors on the macrophage surface, since a number of other factors, such as susceptibility and resistance of the host source of cells and previous exposure of the host to the parasite, did not influence the phagocytic ability of the macrophages. The level of expression of a number of molecules on the surface of macrophages have been used to differentiate macrophage populations (Leenen *et al*, 1986; Sato and Hughes, 1994;). For example, the Mac-1 antigen is the complement receptor type 3 (CR3), and is a member of the integrin family. It is expressed on monocytes/macrophages, neutrophils, and natural killer cells (NK). The Mac-2 antigen, a member of the 30-35 kDa lectin family, is expressed on mast cells, neutrophils and eosinophils (discussed in Sato and Hughes, 1994). The mannose fucose receptor (MFR) also known as the macrophage mannose receptor (MMR) is expressed on macrophages and hepatic endothelial cells (Stahl, and Gordon, 1982). The FcRII is expressed on monocytes/macrophages, eosinophils, neutrophils, basophils and lymphocytes (Unkeless, 1979). ICAM-1 (intercellular adhesion molecule-1) is expressed by endothelial cells, lymphocytes, monocytes and macrophages, fibroblasts and keratinocytes (Dustin *et al*, 1986).

Different surface receptors are not exclusively expressed on macrophages, and are not all directly involved in phagocytosis; the CR3, MFR and FcRII are phagocytic receptors. Other macrophage surface receptors may play a role in phagocytosis through binding of the parasite to the macrophage surface. This may have occurred with the *scid*-source amastigotes. BMDM were not as susceptible to these amastigotes after a 2 hr incubation compared with BALB/c-source amastigotes. However, after 24 hr the numbers of macrophages infected with BALB/c-source or the *scid*-source of amastigotes were similar. This occurred even after washing the macrophages to remove free amastigotes, implying that the *scid*-source amastigotes remained bound to the macrophage surface and were phagocytosed at a later date possibly following turnover of the particular phagocytic receptors. The high specificity of *Leishmania* for their host cell, the macrophage, implies that there must be a combination of specific receptors that target the parasite to the cell and induce phagocytosis by the macrophage.

All the primary macrophage populations and the mature macrophage cell lines that I studied express the CR3 (mac-1), FcRII, mac-2, mac-3 and ICAM-1 (Leenen *et al.*, 1986; Goebler *et al.*, 1993; Ralph *et al.*, 1983; Cox *et al.*,, 1989; Haeffner-Cavaillon *et al.*, 1979; Leenen *et al.*, 1994; Walker *et al.*, 1985;

Schreiber *et al*, 1990; Ho and Springer, 1992). The MFR, expressed on BMDM and peritoneal cells is not expressed by the macrophage cell lines J774A.1 and P388D.1 (Stahl and Gordon, 1982). Levels of expression of these receptors differs in the macrophage populations examined in this study. Resident and thioglycolate elicited peritoneal cells obtained from BALB/c mice express similar levels of mac-3 antigen and FcRII, while mac-1 expression is higher in the resident population and mac-2 expression is higher in the inflammatory population (Leenen *et al.*, 1986). Ho and Springer (1982) reported equivalent expression of mac-1 on resident and thioglycolate elicited macrophages obtained from C57BL/c mice, indicating that receptor expression may be dependent on the strain of mouse from which the cells are obtained. However, I did not see evidence for this when comparing phagocytosis by BMDM from different strains of mice.

Peritoneal macrophages phagocytized few promastigotes in this study, whereas the same cells phagocytized more BALB/c and *scid*-source amastigotes. The entry of BALB/c-source amastigotes into peritoneal cells has been previously studied. Fortier *et al.* (1982) showed that the young, peroxidase positive, subpopulation of inflammatory and resident macrophages phagocytosed more *L. major* amastigotes than the mature macrophages. Pappas (1983) reported that *L. major* grows better in the inflammatory cells than resident peritoneal macrophages. *L. donovani*, on the other hand, is phagocytosed by peritoneal cells, but, barely survives following internalization (Crocker *et al.*, 1987).

The four mature macrophage cell lines I examined, were similar in their susceptibility to the parasite. Not surprisingly, the myeloblast cell line, M1, and the immature macrophage line WEHI-3 were extremely resistant to the parasite since these cells express very little or no CR3 and FcRII. The cells that were infected appeared morphologically different than the non-infected cells. These may be cells that were more mature and expressing more receptors that participate in phagocytosis. Under certain conditions these lines can be stimulated to express complement and Fc receptors (Ralph *et al.*, 1983).

A handful of studies have examined the cells at the lesion site during cutaneous leishmaniasis. Macrophages in the footpads of BALB/c mice infected with *L. major* express both the CR3 and FcRII (Heinzel *et al.*, 1988). When *L. major* is injected subcutaneously into mice, the cells that accumulate in the liver are highly mac-1 positive (Davies *et al.*, 1988). In ear

tissue the majority of cells are macrophages that are ICAM-1 positive (Goebeler *et al.*, 1993). Promastigotes of *L. major* increase ICAM-1 expression on macrophages when incubated with macrophages for 24 hours in vitro (Goebeler *et al.*, 1993). Therefore, the expression of the phagocytic macrophage receptors CR3 and FcRII on cells in the lesion suggests that they may be important in phagocytosis of the parasite. ICAM-1 may be involved in binding the parasite to the macrophage.

When amastigotes of *L. major* are injected into the liver of mice the parasites preferentially infect infiltrating macrophages, unlike *L. donovani* that infects mainly the resident population (Davies *et al.*, 1988). Macrophages that are recruited to the lesion site in *L. donovani* infected mice may be important in clearing the infection because mice treated with anti-CR3 mAb 5C6 have reduced cell recruitment and prolonged infection (Cervia *et al.*, 1993). In addition, *L. major* induces a higher influx of cells into the liver than *L. donovani*. Myelopoeisis is increased 60-fold in the spleens of BALB/c mice infected subcutaneously with *L. major* (Mirkovich *et al.*, 1986). Thus, the two species of *Leishmania* infect different macrophage populations, *L. major* preferentially infecting the inflammatory macrophages.

The differences observed in the phagocytosis of the two stages of parasite and the two sources of amastigotes imply differences in externally exposed parasite ligands and host components. Moreover, heterogeneity of the macrophage populations was evident and implies differences in receptor expression on the macrophage surface. The next chapter describes the results of studies that identified the macrophage receptors used for entry of *Leishmania* amastigotes into its host cell.

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Chapter V: Macrophage Receptors Required for Entry of Amastigotes of *Leishmania major* Into the Host Cell. [1]

INTRODUCTION

Numerous studies have identified macrophage receptors involved in the entry of *Leishmania* promastigotes. These receptors include: the mannose fucose receptor (MFR) (Blackwell *et al.*, 1985; Channon *et al.*, 1984; Wilson & Pearson, 1986, 1988), the receptor for advanced glycosylation end products (AGE) (Mosser *et al.*, 1987), the fibronectin receptor (Rizvi *et al.*, 1988; Wyler *et al.*, 1985), the Fc receptor (Chang, 1981; Russell *et al.*, 1989) and the complement receptors CR1 and CR3 (Blackwell *et al.*, 1985; Cooper *et al.*, 1988; Da Silva *et al.*, 1989; Mosser & Edelson, 1984; 1985; 1987; Russell & Wright, 1988; Talamas-Rohana *et al.*, 1990; Wilson & Pearson, 1988).

Despite the wealth of information about the receptors used for entry of the promastigote stage very little is known about the mechanisms of entry of amastigotes which are responsible for the disease in mammalian hosts. In addition, studies to date did not examine receptor-mediated entry of both stages of the same species of *Leishmania*. The aim of the research presented in this chapter was to use a comparative approach to examine the mode of entry of promastigotes and amastigotes of a single species of *Leishmania*, *L. major*, in an attempt to identify macrophage receptors utilized by the parasite to enter the host cell. The results suggest both differences and similarities in the macrophage receptors utilized by the insect and tissue stages of *L. major*. In addition, differences in the host cell-parasite interactions were observed between two sources of amastigotes, from the BALB/c and *scid* mice.

RESULTS

The Fc receptor (FcR):

Fc receptor involvement in entry of *L. major* was studied by quantifying the entry of parasites opsonized with parasite-specific IgG into macrophages. The parasite-specific IgG was purified from the serum of

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[1] A version of this chapter is published: Guy and Belosevic. Infect. and Immun. 1993. 61:1553-8.

BALB/c mice infected with *L. major*. Opsonization of promastigotes with specific antibody, but not normal IgG, significantly enhanced their uptake into macrophages, as shown by the increase in the percent infected macrophages (Figure 5-1), and higher numbers of promastigotes per macrophage. An average of 200 ± 3 parasites per 100 macrophages were seen in the absence of antibody or when normal IgG was present, whereas 280 ± 8 parasites per 100 macrophages were observed in the presence of parasite-specific IgG. Maximal enhanced entry was seen with 25 μg/ml of anti-*L. major* antibody and similar results were obtained with macrophages in suspension or adhered to glass slides.

Unlike promastigotes, amastigote entry was not enhanced in the presence of parasite-specific IgG (Figure 5-1). Amastigotes obtained from the footpads of BALB/c mice; however, have antibody bound to their surface. In an IFA, the entire surface of the amastigote fluoresced when the reaction was performed at 4°C and capping of the antibody occurred at 37°C (data not shown). The iso- and sub-isotypes of the surface-bound antibodies were identified by ELISA using amastigotes that were obtained from mice between 28 and 36 days post infection. The most predominant antibody isotype found on the amastigote surface was IgG_1, followed by IgM, IgG_{2a} and IgG_{2b} in similar proportions (Figure 5-2). Thus, it would appear that the surface of the parasite is saturated with immunoglobulin, which may explain why I did not observe enhanced entry of amastigotes following addition of exogenous parasite-specific IgG to the cultures.

To determine the importance of the Fc receptor in entry I first attempted to remove bound Ig from the surface of amastigotes obtained from BALB/c mice by chemical treatment. Antibody remained surface bound following treatment of amastigotes with 0.01% sodium dodecyl sulfate (SDS) or 168 mM NH_4Cl (pH 5.0), detected by IFA and ELISA. Treatments with 0.01 M acetic acid in water (pH 3.0) or PBS (pH 3.9), which is used to separate antigen-antibody complexes, killed the amastigotes. Two alternative procedures to obtain amastigotes free of host immunoglobulin (Ig) were developed. The first source of Ig-free amastigotes was bone marrow-derived macrophages (BMDM) infected in vitro with promastigotes of *L. major*. Nine to 12 day old macrophages were infected and cultured at 37°C for an additional 10 to 12 days to allow the promastigotes to transform into amastigotes and for amastigote replication to occur. The amastigotes were

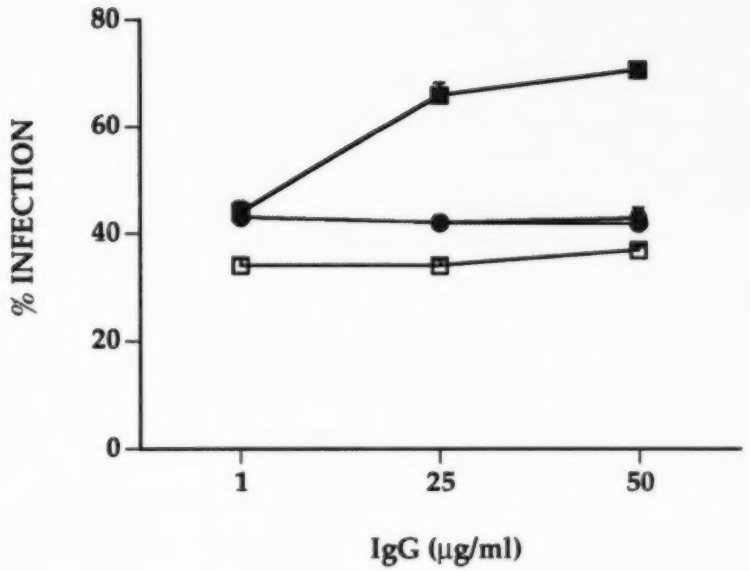

Figure 5-1: The entry of promastigotes (squares) and amastigotes of *L. major* (circles) opsonized with parasite-specific IgG into macrophages. Open symbols represent parasites opsonized with normal Ig and the closed symbols represent parasites opsonized with anti-*L. major* IgG. A ratio of 2:1 parasites to macrophages was used. The amastigotes were obtained from the footpads of BALB/c mice. Results were expressed as the mean ± S.E.M. of three replicates per group. These data are representative of two experiments with similar results.

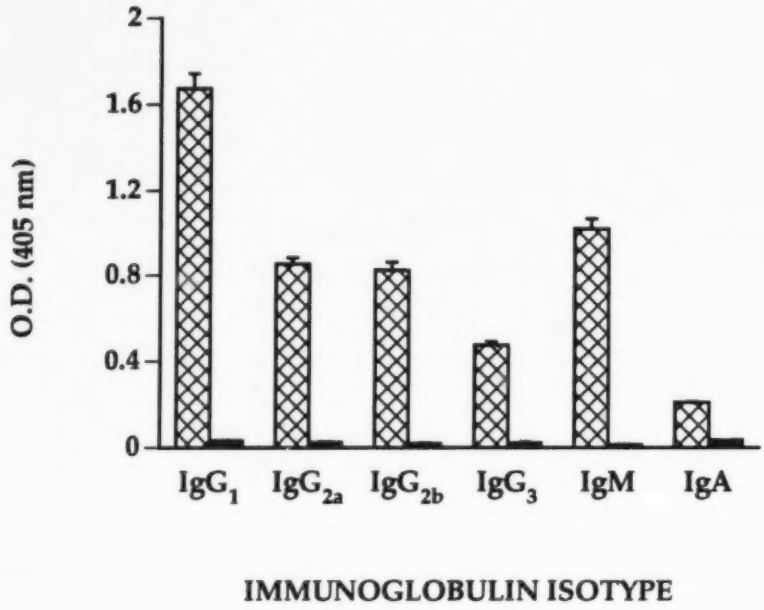

Figure 5-2: Isotype and subisotype distribution of anti-*L. major* antibodies on the surface of amastigotes isolated from the footpads of BALB/c (hatched bars) and *scid* (solid bars) mice. Shown are the mean optical density (O.D.) values ± S.E.M. of amastigotes, obtained from 5 animals per strain of mouse, reacted in an ELISA. The data are representative of 5 experiments with similar results.

liberated from the infected cells by lysing macrophages with 0.01% sodium dodecyl sulfate and washed, at 800 x *g* for 10 min., prior to use in the assays. The second source of Ig-free amastigotes was *scid* mice infected with *L. major*. *Scid* mice lack the ability to produce Ig and the amastigotes from these mice do not have surface bound Ig as shown by ELISA (Figure 5-2). Using these amastigotes, I observed a significant enhancement of parasite entry in the presence of parasite-specific IgG (Figure 5-3). Opsonization of the amastigotes obtained from in vitro culture and *scid* mice enhanced uptake of the parasite by 40% and 73%, respectively. These results indicate that the Fc receptor is important for the entry of amastigotes into macrophages.

Binding to the FcR and MFR triggers respiratory burst activity whereas binding to the CR1 and CR3 does not (Wright and Silverstein, 1983). It is possible that the FcR would not be an appropriate receptor for amastigotes to use because they would be subjected to the toxic molecules generated by the respiratory burst activity. However, in these studies, amastigotes obtained from either BALB/c or *scid* mice survived within macrophages in vitro for at least 72 to 96 hours. In addition, amastigotes from *scid* mice, opsonized with IgG, survived for 72 hours within BMDM in vitro. The infection levels in BMDM in vitro, 72 hours after exposure to amastigotes from *scid* mice, were 70%, 80% and 87% for unopsonized amastigotes, IgG opsonized and complement opsonized parasites, respectively. Therefore, amastigotes entering by the FcR survive within the macrophage.

As was seen in the previous chapter (Chapter IV, Figure 4-1), the uptake of amastigotes from *scid* mice is not as efficient, over a 2 hr. period, when compared to amastigotes from BALB/c mice. To obtain an infection rate of 50%, a 2: 1 amastigote:macrophage ratio is required when using amastigotes from BALB/c mice. On the other hand, a 10:1 ratio is necessary to obtain 50% infection using amastigotes from *scid* mice. Since a 50% infectivity was required for the inhibition of entry bioassay, all further experiments were performed at a parasite to macrophage ratio of 2:1 for amastigotes obtained from BALB/c mice and 10:1 for amastigotes from *scid* mice and stationary phase promastigotes obtained from in vitro culture.

The importance of Fc receptors in entry of *Leishmania* was next examined by down-modulating the receptors onto immune complexes following the procedure of Michl *et al.* (1979). The macrophages were adhered to the coated slides for one hour prior to exposure to amastigotes. As expected,

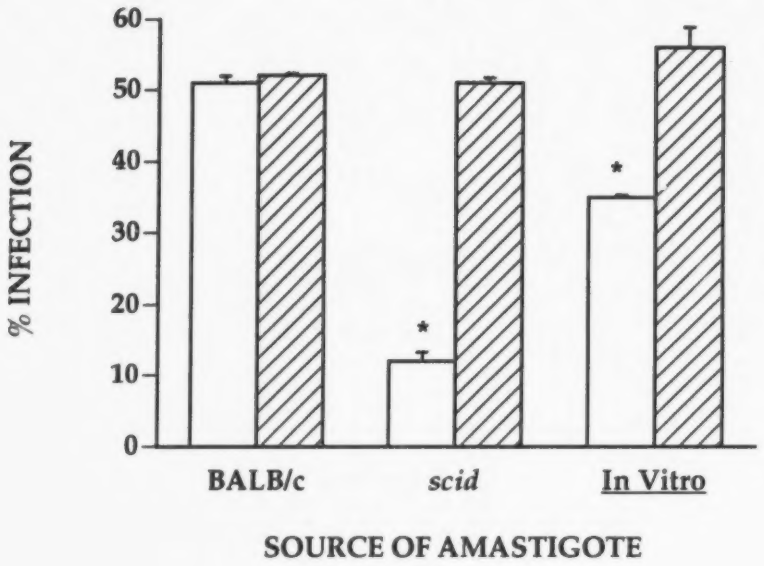

Figure 5-3: The entry of amastigotes of *L. major*, opsonized with normal IgG (hollow bars) or parasite-specific IgG (diagonal slashed bars), into macrophages. The ratio of amastigotes to macrophage was 2:1. The source of amastigotes were from the footpads of either BALB/c or *scid* mice, or from BMDM in vitro. Results were expressed as the mean percentage of infected macrophages ± S.E.M. of three replicates per group. These data are representative of at least two experiments with similar results. (*) Significantly different ($p<0.03$) than cells opsonized with parasite-specific IgG.

no change in the percent of macrophages infected was observed with amastigotes from *scid* mice when they were adhered to immune complexes as compared to those adhered to the individual components of the complex, either DNP or anti-DNP (Table 5-1). Surprisingly, only a slight but not significant decrease occurred in the uptake of amastigotes from BALB/c mice into macrophages adhered to complexes (Table 5-1). In addition, there was no reduction in entry of amastigotes from *scid* mice that were opsonized with parasite-specific IgG into macrophages adhered to immune complexes, whereas phagocytosis of Ig-opsonized sheep erythrocytes was significantly reduced, by 92%. Michl *et al.* (1979) demonstrated that the Fc receptor for IgG_{2a} is not down modulated onto complexes and that treatment of macrophages with trypsin destroys this receptor. To ensure that amastigotes were not entering by binding the FcγRI the macrophages were treated with trypsin (Table 5-1). No reduction in the uptake of the amastigotes was observed, indicating that the FcgRI for IgG_{2a} is not required for entry of amastigotes.

In addition to using immune complexes to down modulate Fc receptors I employed the mAb 2.4G2 which recognizes the FcγRII. Culturing macrophages on up to 100 µg/ml of mAb 2.4G2 coated slides did not alter the uptake of amastigotes obtained from either strain of mouse, whereas uptake of Ig-opsonized sheep red blood cells was reduced by 43%. Similarly, the addition of soluble mAb 2.4G2 had no effect on the uptake of amastigotes into macrophages.

The complement type 3 receptor (CR3):

Complement receptor-mediated entry of *Leishmania* into macrophages has been well documented for promastigotes but not amastigotes. I examined the use of complement receptors by blocking the receptors with mAbs. A mAb against the surface antigen PGP-1 (CD44), which bound to the surface of the macrophages, observed using IFA, was used as a control. In general, mAbs against the α- and ß- chains of CR3 were effective in reducing amastigote entry, suggesting that the CR3 is utilized by the tissue form of *L. major* for entry into its host cell. It should be noted that the degree of inhibition of entry differed among different macrophage populations. For example, the addition of anti α-chain mAb (M1/70) inhibited entry of amastigotes only in resident peritoneal cell (rPC) and J774A.1 cells, a 42% and 28% reduction in this particular experiment (Table 5-2). In contrast, the addition of anti-ß-chain

Table 5-1: The percent infection of macrophages adhered to immune complexes, with amastigotes obtained from different sources.

TREATMENT	AMASTIGOTE	
	BALB/c	*scid*
DNP	74 ± 2	59 ± 3
Anti-DNP	68 ± 4	57 ± 2
Immune Complex	67 ± 2	63 ± 1
Trypsinization	62 ± 1	60 ± 3
Immune Complex + Tryprsinization	58 ± 3	64 ± 5

The macrophages were adhered to coated slides for 1 hr. prior to exposure to amastigotes. Data are presented as the mean ± S.E.M. of 4 replicates per treatment and are representative of 3 experiments with similar results.

Table 5-2: The percent infection of macrophages and reduction the in entry of promastigotes and BALB/c-source amastigotes into macrophages in the presence of anti-CR3 mAbs.

mAb	MACROPHAGE POPULATION							
	BMDM		rPC		iPC		J774A.1	
	% Inf.	% Red.	% Inf.	% Red.	% Inf.	% Red.	% Inf.	% Red.
AMASTIGOTES								
PGP-1	61 ± 2.3		25 ± 1.3		40 ± 0.3		54 ± 0.7	
M1/70	58 ± 0.3	(5%)	15 ± 3.2 *	(42%)	38 ± 1.7	(5%)	39 ± 0.3 *	(28%)
M18/2	36 ± 0.7 *	(41%)	11 ± 1.8 *	(58%)	30 ± 0.0 *	(25%)	48 ± 1.5	(11%)
PROMASTIGOTES								
PGP-1	63 ± 0.9		13 ± 0.6		17 ± 1.0		55 ± 3.0	
M1/70	61 ± 1.0	(3%)	13 ± 0.7	(0%)	17 ± 1.5	(0%)	53 ± 1.2	(4%)
M18/2	57 ± 0.7	(10%)	4 ± 0.6 *	(69%)	14 ± 1.7	(18%)	56 ± 2.0	(0%)

% Inf. = percent of infected macrophages; % Red.= percent reduction compared to control mAb PGP-1.
The macrophage populations were: bone marrow derived macrophages (BMDM); resident peritoneal cells (rPC); inflammatory peritoneal cells (iPC); the cell line J774A.1. The mAbs: PGP-1 a control mAb that binds to the surface of the macrophage; M1/70 recognizes the α-chain of the CR3; M18/2 recognizes the β-chain of the CR3. Each treatment was done in triplicate and is representative of at least 2 experiments with similar results.
(*) Significantly different ($p<0.03$) compared to treatment with the control mAb PGP-1.

mAb (M18/2) to BMDM and rPC cultures reduced the entry of BALB/c source amastigotes by 41% and 58%, respectively, but in J774A.1 and inflammatory PC (iPC) the reduction in entry varied from experiment to experiment and was never greater than 25% (Table 5-2). The entry of promastigotes in the absence of complement was not inhibited by mAbs M18/2 and M1/70, with one exception: MAb M18/2 significantly reduced uptake of promastigotes into rPC (Table 5-2). Interestingly, mAb M18/2 did not reduce uptake of promastigotes into BMDM; however, amastigote entry was reduced with the same mAb into BMDM.

A dose response curve indicated that 25 µg/ml gave maximal inhibition and thus was the concentration used in the assays. This degree of inhibition was not enhanced by increasing the concentration of mAb, altering the time of pre-incubation of macrophages with the mAb, the incubation temperature, or by down-modulating the CR3 by adhering macrophages onto mAb-coated slides.

A comparison of the blocking of entry of the two sources of amastigotes revealed similar results. The anti-CR3 mAb was equally effective in blocking entry of the *scid*-source amastigotes as the BALB/c-source, despite the differential amastigote:macrophage ratios used for amastigotes from BALB/c and *scid* mice, 2:1 and 10:1 respectively (Table 5-3). A significant reduction in entry was consistently found, ranging from 25 to 40% with mAb M1/70 into rPC and J774A.1 cells. M18/2 reduced entry of both sources of amastigote into macrophages (Table 5-3).

A different pattern of blocking was observed with promastigotes when fresh mouse serum was added to the cells, whereas fresh serum did not alter the blocking of amastigotes entry into macrophages by the anti-CR3 mAbs. It should be noted that the addition of fresh normal mouse serum significantly enhanced promastigote entry, in the absence of blocking mAbs, but did not affect the uptake of the amastigotes (Table 5-4). MAb M1/70 significantly decreased the uptake of the promastigotes into the macrophages when 10% fresh serum was added to the cultures (Table 5-4). No significant decrease with mAb M1/70 was observed in the absence of serum (Tables 5-2 and 5-4). On the other hand, the blocking of promastigote entry into macrophages observed with mAb M18/2 (Table 5-2 and 5-4) was abrogated when fresh serum was added to the cells.

Table 5-3: The percent infection and the reduction in entry of amastigotes from BALB/c and *scid* mice in the presence of anti-CR3 mAbs.

MAb	AMASTIGOTE			
	BALB/c		*scid*	
	% Infection	% Reduction	% Infection	% Reduction
PGP-1	17 ± 1.2		37 ± 2.4	
M1/70	12 ± 2.7	31	25 ± 1.2 *	34
M18/2	6 ± 1.2 *	66	15 ± 1.5 *	60

The reduction in entry is the difference between the entry of anti-CR3 mAbs compared to the control mAb PGP-1. The data are presented as the mean ± S.E.M. Each treatment was performed in triplicate and the data are representative of 2 or more experiments with similar results.
(*) Significantly different ($p<0.001$) from the control mAb PGP-1.

Table 5-4: The effects of mouse serum on the ability of mAbs to block entry of promastigotes and BALB/c-source amastigotes into macrophages.

AMASTIGOTES

MAB	PERCENT INFECTION OF MACROPHAGES 10% MOUSE SERUM		
	None	Normal	Heat-Inactivated
PGP-1	26 ± 1.5	26 ± 1.2	24 ± 2.0
M18/2	11 ± 1.2 *	11 ± 0.9 *	7 ± 0.6 *
M1/70	16 ± 0.9 *	12 ± 0.7 *	10 ± 2.2 *

PROMASTIGOTES

MAB	PERCENT INFECTION OF MACROPHAGES 10% MOUSE SERUM		
	None	Normal	Heat-Inactivated
PGP-1	5 ± 0.7	73 ± 2.6	5 ± 0.9
M18/2	3 ± 0.6	69 ± 1.5	4 ± 0.7
M1/70	4 ± 0.3	31 ± 0.3 *	6 ± 1.0

The macrophages were pre-incubated with 25 µg/ml mAb. After 30 min., fresh mouse serum was added at a final concentration of 10%, followed by the parasite. The infection ratio was 2:1 for amastigotes and 10:1 for promastigotes. The data are presented as the mean ± S.E.M. Each treatment was performed in triplicate and the data are representative of 2 experiments using resident peritoneal cells, both with similar results.
(*) Significantly different ($p<0.001$) compared to mAb PGP-1.

When the cells were opsonized with guinea pig serum (GPS) as the source of complement, at a dilution of 1:100, there was a 23% increase in the uptake of the promastigotes and a 68% increase in the level of infection of *scid* source amastigotes. Heat inactivation of GPS abrogated this effect. On the other hand, there was a significant decrease in the uptake of BALB/c source amastigotes at a 1:100 dilution of GPS, but, heat treated BALB/c source amastigotes entered macrophages at the same percent as untreated controls. The viability of amastigotes from BALB/c mice, in the presence of fresh GPS, was decreased by 91%, whereas promastigote viability only decreased by 13% compared to untreated parasites.

An examination of the surface of the parasites for the presence of complement component C3 revealed that amastigotes obtained from the footpads of BALB/c mice possess host complement components on their surface in addition to having surface bound Ig (Figure 5-4). The level of complement components was much higher on amastigotes from BALB/c mice than *scid* mice or promastigotes. All stages of the parasite were capable of binding exogenous C3 as can be seen when GPS was added to the parasites in the ELISA.

To examine the role of complement receptors further, macrophages were treated with the synthetic microtubule inhibitor nocodazole (NOC) and the uptake of amastigotes was quantified (Table 5-5). Newman *et al.* (1991) demonstrated a reduced ability of NOC-treated macrophages to phagocytose C3b- and C3bi-opsonized sheep red blood cells, whereas phagocytosis of IgG-opsonized cells was unaffected. I found a significant reduction in the uptake of amastigotes from *scid* mice into NOC-treated macrophages and only a slight reduction in the uptake of amastigotes from BALB/c mice (Table 5-5).

The compounds diacylglycerol (OAG) and phorbol myristate acetate (PMA) were also tested for their ability to enhance uptake of amastigotes into macrophages. OAG enhances phagocytosis through both the CR3 and FcRs and PMA enhances CR3 mediated phagocytosis. Neither compound altered the ability of macrophages to phagocytose amastigotes obtained from either BALB/c or *scid* mice when preincubated with macrophages at concentrations of 1, 10, 50, 100 and 150 µg/ml for OAG and 0.5, 1, 5, 10, 20 or 30 ng/ml for PMA.

Several binding sites have been identified on CR3, and besides the site for C3bi binding there is an LPS binding site (Wright and Jong, 1986).

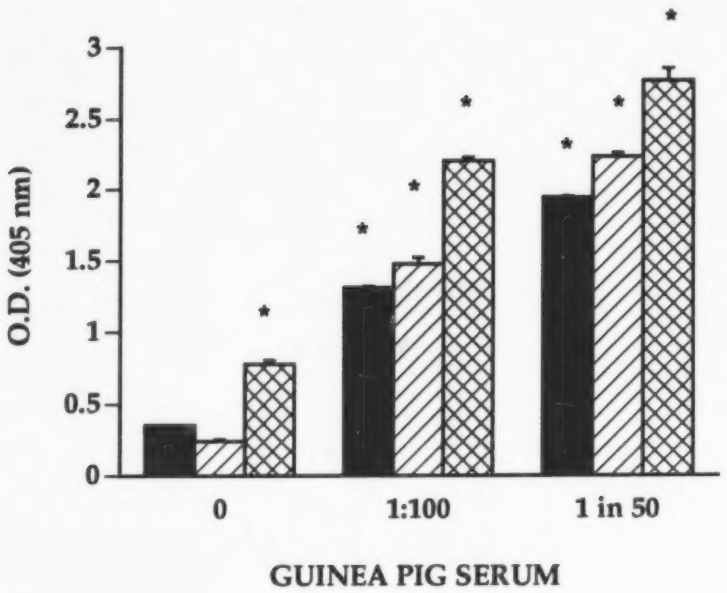

Figure 5-4: Binding of anti-human C3 antibody to promastigotes and amastigotes obtained from BALB/c or *scid* mice. The solid bars represent promastigotes, slashed bars the *scid*-source amastigotes and hatched bars the BALB/c-source amastigotes. Shown are the mean optical density (O.D.) values of promastigotes or amastigotes (obtained from 5 animals in the *scid* mouse group and 2 animals in the BALB/c group) reacted in an ELISA. The data are presented as the mean ± S.E.M. and the data are representative of 3 separate experiments with similar results. (*) Significantly different ($p<0.001$) than other parasites.

Table 5-5: The percent infection of amastigotes in macrophages treated with nocodazole.

TREATMENT	AMASTIGOTE	
	BALB/c	*scid*
	% Infection	% Infection
NOC (μg/ml)		
0	64 ± 1	40 ± 2
2.5	52 ± 3	33 ± 1
5.0	53 ± 1	21 ± 2 *

The macrophages, adhered to glass slides, were treated for 30 min. with nocodazole (NOC) prior to exposure to the amastigotes. Control wells received 1% DMSO in DMEM, which was used to dissolve the NOC. The data are representative of 3 experiments with similar results.
(*) Significantly different ($p<0.01$) compared to 0 μg/ml NOC.

However, the LPS site does not appear to be utilized by *L. major* promastigotes or amastigotes from BALB/c mice because preincubation of macrophages with lipopolysaccharide (LPS) from *Eishcherichia coli* J5 Rc strain did not alter uptake at concentrations ranging from 1 to 30 μg/ml. In addition, no effect was observed with LPS obtained from *Salmonella typhimurium*.

Since blocking of the FcR did not inhibit entry of the parasite, it appears that amastigotes have the ability to change the receptors they bind. To address this issue, the FcR and CR3 were blocked simultaneously. Interestingly, when macrophages were adhered to immune complexes and then treated with either mAb M1/70, NOC or a combination of both, I found a significant reduction in entry of amastigotes from either BALB/c or *scid* mice (Table 5-6).

The mannose fucose receptor (MFR):

The use of the MFR was examined by blocking the receptor with the specific oligosaccharide, mannan. Preincubation of macrophages with soluble mannan at concentrations from 1 to 30 mg/ml did not alter the uptake of either promastigotes or amastigotes from BALB/c or *scid* mice. The importance of the MFR for parasite entry was further examined by down-modulating the MFRs onto mannan coated glass slides. No inhibition was observed in the uptake of amastigotes from either BALB/c or *scid* mice, when adhered macrophages were treated with up to 10 mg/ml of soluble mannan. Similarly, the entry of amastigotes into macrophages adhered to mannan coated glass slides (1 to 10 mg/ml mannan) was not inhibited. These results suggest that the MFR is not the primary receptor for the entry of amastigotes of *L. major*.

Interestingly, a number of monosaccharides did alter the ability of the promastigote stage to enter macrophages in vitro. It should be noted that this inhibition of entry was dependent upon the concentration of the inhibitor since the inhibition diminished with decreasing concentrations of carbohydrates (Table 5-7). The viability of macrophages and parasites was unaltered at the high carbohydrate concentrations as evidenced by Trypan blue dye exclusion (Figure 5-5). The concentrations of carbohydrates that resulted in the greatest inhibition of entry are shown in Table 5-8. Treatment of macrophages with N-acetyl glucosamine, L-fucose, mannose, and glucose significantly inhibited entry of promastigotes into macrophages, as evidenced by fewer infected macrophages and fewer parasites per macrophage; the

Table 5-6: The percent infection of amastigotes in macrophages adhered to immune complexes.

TREATMENT	BALB/c		*scid*	
	% INFECTION	% INHIBITION	% INFECTION	% INHIBITION
Irrelevant mAb	48 ± 1	0	63 ± 5	0
mAb M1/70	32 ± 3 *	38	44 ± 1 *	30
NOC	25 ± 2 *	50	32 ± 1 *	49
M1/70 + NOC	19 ± 2 *	60	19 ± 1 *	70

The percent infection of macrophages adhered to immune complexes but in the absence of treatment was: 46 ± 4% with amastigotes from BALB/c mice and 61 ± 2% with amastigotes from *scid* mice. The data represents the mean ± S.E.M. of four replicates per group. Macrophages were adhered to immune complexes then preincubated for 30 min. with inhibitors prior to a one hour exposure to amastigotes. MAbs were added at a concentration of 25 μg/ml and nocodazole (NOC) at 5 μg/ml. (*) Significantly different ($p<0.001$) compared to control mAb PGP-1.

Table 5-7: A dose response of the effects of carbohydrates on the entry of promastigotes into macrophages.

CARBOHYDRATE (mM)	% INFECTION	% REDUCTION
Medium	40 ± 1.0	
N-acetyl glusosamine		
25	31 ± 1.5 *	23
50	30 ± 3.0 *	25
100	24 ± 1.7 *	40
Fucose		
100	37 ± 1.3	8
200	28 ± 1.3 *	23
300	26 ± 1.4 *	35
Glucose		
50	30 ± 2.4 *	25
100	32 ± 2.0 *	20
200	27 ± 3.0 *	33

The data are presented as the mean ± S.E.M. Each treatment was performed in triplicate and are representative of at least 3 experiments with similar results. (*) Significantly different ($p<0.002$) compared to medium.

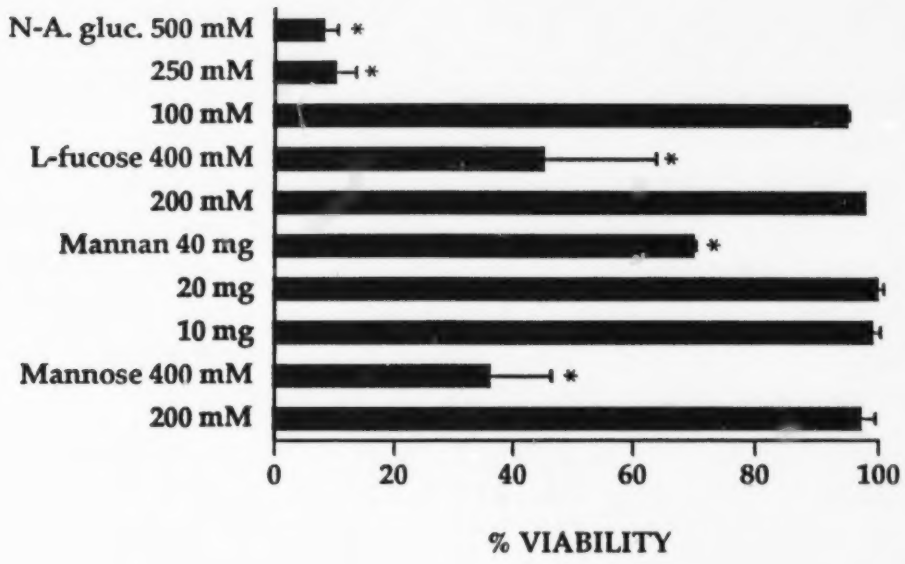

Figure 5-5: The viability of macrophages after treatment with different carbohydrates. The macrophages were treated for 30 min. then stained with trypan blue and the number of viable cells was determined. The data is represented as the mean ± S.E.M. of the percent of viable cells compared to untreated macrophages. Each treatment was performed in triplicate and is representative of 2 experiments. (*) Significantly different ($p<0.02$) from medium control.

Table 5-8: The percent infection of macrophages with promastigotes and BALB/c-source amastigotes in the presence of carbohydrates.

MACROPHAGES	PROMASTIGOTES		AMASTIGOTES	
TREATMENT	% Inf.	% Red.	% Inf.	% Red.
Medium	40 ± 1.0		46 ± 2.1	
100mM N-acetyl glucosamine	24 ± 1.7 *	40	42 ± 0.7	9
200mM L-fucose	28 ± 1.3 *	30	44 ± 2.2	4
5 µg/ml mannan	37 ± 1.6	8	47 ± 0.7	0
200mM mannose	31 ± 3.4	23	45 ± 1.9	2
Medium	44 ± 1.5		48 ± 1.4	
40 µg/ml ß-glucan	44 ± 1.7	0	47 ± 2.6	2
200mM glucose	28 ± 0.9 *	36	46 ± 1.5	4
100mM maltose	45 ± 0.9	0	47 ± 3.5	2

% Inf. + percent infected macrophages. % Red. = percent reduction. The BMDM were preincubated with carbohydrate for 30 min. at 37°C prior to exposure to the parasite for 2 hrs. Promastigotes were obtained from in vitro culture, during the stationary phase, and amastigotes were obtained from the BALB/c mouse. Results are expressed as the mean percent infection of 3 replicates per group ± S.E.M. The data represents one of three experiments with similar results. (*) significantly different ($p<0.01$) from medium control.

specific inhibition ranged from 23-40%. In control cultures there were 229 ± 6.5 parasites per 100 macrophages, whereas in the presence of N-acetyl glucosamine, L-fucose, mannose, and glucose, infection levels were 172 ± 3.2, 165 ± 2.7, 177 ± 5.8 and 161 ± 0.6, respectively. Mannan, ß-glucan or maltose did not alter the entry of promastigotes. On the other hand, the percentage of macrophages infected with amastigotes from BALB/c mice and the number of amastigotes per macrophage were not affected by the addition of specific carbohydrates to macrophage cultures.

Two carbohydrates that blocked entry of promastigotes into BMDM also reduced the entry of amastigotes from *scid* mice, entry decreased by 29% and 45% in the presence of N-acetyl glucosamine and glucose (Figure 5-6). This effect was abrogated when the *scid* amastigotes were opsonized with either parasite-specific IgG or GPS prior to exposure to the carbohydrate treated macrophages (Table 5-9). Therefore, the *scid* amastigotes behaved like the BALB/c amastigotes following opsonization.

From these studies it appears that although the MFR is not involved in entry there is some interaction between carbohydrates on the parasite surface and macrophage receptors. In addition, this interaction can be disrupted and the route of entry altered, as seen with opsonization of the *scid* amastigotes.

DISCUSSION

Receptor-mediated entry of intracellular pathogens into macrophages has received much attention in the last 15 years. Although differences are apparent between the organisms in the types of receptors used and the requirement for serum opsonization, it is evident that many intracellular pathogens use the MFR, FcR or complement receptors for entry into macrophages. For example, the yeast stage of the fungal pathogen *Histoplasma capsulatum* uses the CR3/LFA-1/ p150, 95 family of adhesion molecules to enter human macrophages, the MFR and FcR are not involved, and serum opsonization is unnecessary for entry (Bullock & Wright, 1990). Similarly, the bacterium *Bordetella pertussis* enters human macrophages through a direct interaction between the filamentous hemagglutinin of the bacterium with the macrophage CR3 (Saukkonen *et al.*, 1991; Ishibashi *et al.*, 1994). Binding of *B. pertussis* to the very late antigen-5 (VLA-5) enhances CR3 mediated phagocytosis (Hazenbos *et al.*, 1993). Uptake of the bacterium

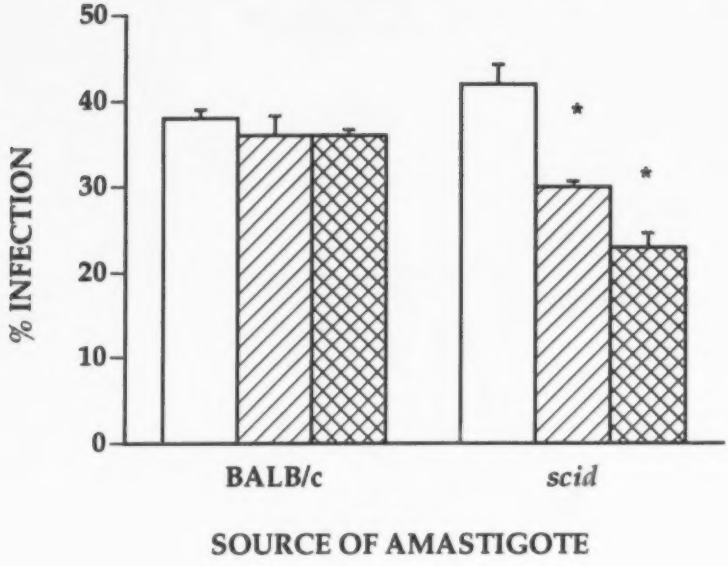

Figure 5-6: The effects of carbohydrates on the entry of *L. major* amastigotes into macrophages. The various treatments were: medium only (hollow bars), 100 mM N-acetyl glucosamine (vertical slashed bars) and 200 mM glucose (hatched bars). The data is presented as the mean ± the S.E.M. of treatments performed in triplicate and is representative of 4 experiments with similar results. (*) Significantly different ($p<0.001$) from the medium control.

Table 5-9: The effects of opsonization of the *scid*-source amastigotes on the blocking of amastigote entry into macrophages by carbohydrates.

MACROPHAGE TREATMENT	PERCENT INFECTION WITH TREATED AMASTIGOTES					
	2:1 RATIO			10:1 RATIO		
	None	IgG	GPS	None	IgG	GPS
Medium	10 ± 1.6	38 ± 1.5	38 ± 2.5	58 ± 1.5	93 ± 1.0	83 ± 1.9
N-acetyl glucosamine	8 ± 0.6	38 ± 1.2	42 ± 0.1	45 ± 2.7 *	92 ± 1.3	87 ± 0.7
Glucose	4 ± 0.9 *	36 ± 2.1	39 ± 2.4	37 ± 0.7 *	90 ± 2.9	87 ± 0.7

The macrophages were preincubated with carbohydrates for 30 min. prior to addition of amastigotes. N-acetyl glucosamine and glucose were added at concentrations of 100 mM and 200 mM, respectively. The amastigotes were incubated with either a 1:40 dilution of parasite-specific IgG or a 1:100 dilution of guinea pig serum (GPS), for 15 min. at 37°C then washed prior to exposure to macrophages for 2 hr. The data are presented as the mean ± S.E.M. of 3 replicates per group and is representative of 2 experiments with similar results. (*) Significantly different ($p<0.05$) from medium control.

Legionella pneumophila proceeds by the CR1 and CR3; however, unlike *H. capsulatum* and *B. pertussis*, *L. pneumophila* requires opsonization with C3b and C3bi for binding to occur (Payne & Horowitz, 1987). Complement opsonization of HIV-1 and HIV-2 enhances phagocytosis of the virus into macrophages through the CR1 and CR3 (Thieblemont *et al.*, 1993). *Mycobacterium leprae* also requires C3 opsonization for uptake by the CR1, CR3 and CR4 (Schlesinger & Horowitz, 1991; 1994). Both the MFR and CR3 are used in entry by *Mycobacterium avium* and *M. tuberculosis*, and while the presence of serum increases the rate of uptake, unopsonized organisms also enter by binding these receptors (Schlesinger *et al.*, 1990; Bermudez *et al.*, 1991; Schlesinger, 1993; Roecklein *et al.*, 1992; Hirsch *et al.*, 1994). Moreover, purified *M. tuberculosis* surface molecules bind directly to the MFR (Goswami *et al.*, 1994; Schlesinger *et al.*, 1994). An organism that uses the MFR but not the FcR or CR3, is *Pneumocystis carinii* (Ezekowitz *et al.*, 1991; Kruskal *et al.*, 1992).

The involvement of the Fc receptors in entry of *Leishmania* has received little attention. Enhanced entry of promastigotes in the presence of immune serum has been reported (Chang, 1981, Russell *et al.*, 1989). We confirm these findings that the entry of promastigotes in the presence of parasite-specific IgG was enhanced. However, I did not observe increased entry of amastigotes obtained from mouse footpads of BALB/c mice. The initial failure to detect increased uptake of opsonized amastigotes was due to the fact that the surface of parasites isolated from the footpads of mice may already be saturated with the antibodies. Indeed, on the surface of amastigotes obtained from the footpads, I detected IgG_1, IgG_{2a}, and IgG_3, which are known to mediate Fc-dependent phagocytosis. The antibody pattern on the surface of the amastigote is similar to that observed in serum of mice infected with *L. tropica* (Olobo *et al.*, 1980). Host IgG has also been observed on the surface of amastigotes of *L. donovani* isolated from the spleens of infected hamsters (Pearson and Roberts, 1990). It is not surprising, therefore, that the addition of exogenous parasite-specific antibody to the cultures had no detectable effect on the entry of the amastigotes into macrophages. Interestingly, I was able to further opsonize BALB/c amastigotes with rabbit anti-*L. major* amastigote serum, since incubation of the amastigotes with the serum in an ELISA gave higher O.D. values than in the absence of serum. However, this treatment did not enhance the uptake of the amastigotes into macrophages. Therefore, it

would appear that the amount of IgG on the amastigote surface is sufficient to bind the available Fc receptors. It is also likely that certain epitopes on amastigotes are antigenic to rabbits but are not antigenic in mice, which would account for the higher titers observed in the ELISA.

That Fc receptors are important in the uptake of amastigotes is demonstrated by our results which showed enhanced entry of amastigotes into macrophages following addition of exogenous anti-parasite antibody, provided that the parasites were obtained from *scid* mice. To date, three classes or FcγR have been described in mice. The FcRγI binds IgG_{2a} and is a high affinity receptor for monomeric IgG. The FcγRII binds to IgG_{2b} and IgG_1 and is involved in clearing immune complexes and the FcγRIII binds IgG_3 (Unkeless *et al.*, 1988; Fridman *et al.*, 1992). $IgG_{1, 2a}$ and $_{2b}$ subisotypes were located in abundance on the amastigote surface (Figure 5-2), yet blocking their individual receptors did not alter the uptake of amastigotes. Moreover, simultaneous blocking of the FcγR I and II had no effect. A previous study showed that blocking of the FcγRII using mAb 2.4G2 did not reduce the entry of amastigotes obtained from the spleens of hamsters infected with *L. donovani*, into murine resident peritoneal cells (Blackwell *et al.* 1985). It appears that the amastigotes are capable of binding to a different receptor thereby gaining entry into the macrophage. The fact that amastigote entry was reduced, beyond the effects of blocking only the CR3, when both the CR3 and FcRII were simultaneously blocked gives support to this theory.

The role for complement receptors CR1 and CR3 in the uptake of promastigotes of *Leishmania* has been demonstrated (Blackwell *et al.*, 1985; Cooper *et al.*; 1988, Da Silva *et al.*, 1989; Mosser & Edelson, 1984; 1985; 1987; Mosser *et al.*, 1992; Russell & Wright, 1988; Talamas-Rohana *et al.*, 1990; Wilson & Pearson, 1988). Blank *et al.*, (1993) demonstrated that the CR3 is used for uptake of amastigotes of *L. major* into epidermal Langerhan cells. The overall infection in these cells was low and amastigotes do not survive and replicate within these macrophages. Thus, they postulated that the Langerhan cells are important in initiating immune responses in the host. In the present study, I report that amastigotes of *L. major* utilize the CR3, since the entry of the parasites was reduced following the addition of anti-CR3 α-chain mAb to the cultures. It should be noted that the inhibition with the anti-CR3 mAbs was dependent on the macrophage population and that this

dependence on type of macrophages used in the inhibition of entry assays, may explain discrepancies in the results obtained in different laboratories.

There is some controversy as to whether M1/70 can modulate Fc receptors in addition to the CR3. Brown *et al.* (1988) reported a decrease in phagocytosis of Ig-opsonized sheep red blood cells by monocytes in the presence of M1/70, whereas Kimura and Griffin (1992) did not observe inhibition of phagocytosis of Ig opsonized cells by murine peritoneal macrophages. I observed a reduction in entry of amastigotes from *scid* mice; however, lacking surface bound IgG these amastigotes cannot bind to Fc receptors. Therefore, the results indicate that the CR3 was blocked by M1/70.

In addition to blocking with mAbs, I observed a reduction in the phagocytosis of amastigotes with the microtubule inhibitor nocodazole, which inhibits CR3-mediated phagocytosis but not FcR-mediated phagocytosis (Newman *et al.* , 1991). Surprisingly, PMA, a potent stimulator of CR3-mediated phagocytosis, that enhances uptake of C3b and iC3b opsonized sheep red blood cells into human monocytes (Wright and Silverstein, 1982; Newman *et al.*, 1991) did not enhance the uptake of amastigotes. Similarly, the synthetic compound OAG, which is a physiological activator of protein kinase C (PKC) and enhances phagocytosis of C3b, C3bi and IgG-opsonized sheep red blood cells into monocytes (Newman *et al.*, 1991), did not enhance uptake of either the *scid* or BALB/c source amastigotes. It is possible that phagocytosis in the murine macrophage system used in this study is not affected by these two chemicals, whereas complement and Fc receptors on human monocytes are very sensitive to stimulation with PMA and OAG.

A single study has implicated the LPS binding site on CR3 for entry of promastigotes of *L. mexicana.* The uptake of beads coated with LPS, from *E. coli* strain J5 rc, is reduced in the presence of LPG isolated from the surface of *L. mexicana* promastigotes (Talamás-Rohana *et al.*, 1990). In my study, soluble LPS from the rough coat strain of *E. coli* that binds to the CR3 did not alter the uptake of either live promastigotes or BALB/c source amastigotes, suggesting that this binding site on the CR3 is not used by either stage of *L. major.* Alternatively, the LPS, while capable of blocking the binding of a parasite ligand, may be incapable of blocking the uptake of intact parasites.

My finding that the addition of fresh serum was necessary for blocking of the stationary phase promastigotes confirms previous reports (Blackwell *et al.*, 1985; Mosser and Edelson, 1985; Da Silva *et al.*, 1989; Mosser *et al.*, 1992).

Fresh serum had no effect on CR3 mAb blocking of amastigote entry with one exception, blocking by M18/2 was abrogated. The mAb M18/2 binds to the ß-chain of the CR3 which is shared by the molecules LFA-1 (lymphocyte activation antigen-1) and p150/95 or the CR4. A likely explanation is that components in the serum bound to the amastigotes causing an alternative binding site to be utilized, thereby preventing reduction in entry. Thus fresh serum acted as a source of complement component C3 to opsonize entry by the CR3. The addition of fresh serum did not alter BALB/c-source amastigote entry presumably because these amastigotes have C3 on their surface, as shown in the ELISA, when human anti-C3 antibodies were reacted against whole parasites. The highest level of C3 was seen in the BALB/c-source amastigotes. The level of C3 on *scid*-source amastigotes was lower. However, C.B-17 *scid* mice have elevated serum complement activity, measured by hemolysis of sheep red blood cells, compared with normal C.B-17 mice (Shultz *et al.*, 1995). Therefore, the lower amount of C3 on the *scid*-source amastigote seen in this study may be due to less complement fixation in the absence of Ig.

The mannose-fucose receptor (MFR) which recognizes oligosaccharide chains terminating in mannose, fucose or N-acetylglucosamine was the first identified macrophage receptor for the entry of promastigotes. I confirm a previous report that the MFR is not utilized by promastigotes of *L. major* to enter macrophages (Da Silva *et al.*, 1989), despite its involvement in entry of promastigotes of *L. donovani* (Blackwell *et al.*, 1985; Wilson & Pearson, 1986, 1988; Chakraborty & Das, 1988; Palatnik *et al.*, 1989; Basu *et al.*, 1991), *L. mexicana* (Russell & Wilhelm, 1986) and *L. tropica* (Zenian, 1981). This receptor plays little or no role in the entry of the "normal" amastigotes, evidenced by unaltered entry of this stage of the parasite in the presence of the specific receptor blocking carbohydrate, mannan. However, in the absence of opsonizing host components (complement, antibody), carbohydrate binding to macrophages may be utilized by the parasite to enter macrophages as we observed decreased uptake of amastigotes from *scid* mice in the presence of monosaccharides. Macrophages possess several surface exposed lectins that act as receptors for carbohydrates (for a review see Taylor, 1993): the peritoneal macrophage galactose receptor specific for galactose, Mac-2 a 32 kDa S-type lectin specific for β-linked galactose, the β-glucan receptor specific for β-glucan, and sialoadhesin that recognizes the terminal oligosaccharide

sequence NeuNAcα2-3Galβ1-3GalNAc. Only the MFR is capable of mediating phagocytosis. Although the MFR and β-glucan receptor do not participate in entry of promastigotes or amastigotes of *L. major*, other lectins may mediate binding of promastigotes and *scid*-source amastigotes to the macrophage surface.

The present study provides evidence that promastigotes and amastigotes of *L. major* both share and have distinct macrophage receptors for entry into their host cell. The observed differences in the receptors used may reflect the altered environment to which the promastigotes and amastigotes are exposed. The promastigotes which initiate the infection are in an "early" inflammatory environment, whereas the tissue in which the amastigotes reside is an acute inflammatory environment. It is expected that the amastigotes are exposed to a variety of inflammatory molecules and host cells not encountered by promastigotes. This may have caused differential selection pressures for alternate mechanisms of entry of the two stages of *Leishmania* into macrophages. In this study it was observed that both stages use the complement and Fc receptors, but only the promastigotes and amastigotes from immunodeficient mice use carbohydrate binding. Although opsonization with parasite-specific IgG enhanced entry of promastigotes, the Fc receptor is probably not used for entry of promastigotes in vivo, during a primary infection, since no parasite-specific antibody is present at the site of inoculation. This receptor; however, is probably very important for entry of amastigotes in vivo and subsequent growth and development of the leishmanial lesion. In addition to the macrophage receptors tested in this study, it is likely that other, as yet unidentified receptors may be involved in the entry of the amastigotes into macrophages. For example, I found that amastigotes isolated from *scid* mice have no surface bound Ig and very little complement bound to their surface, yet *scid* mice are highly susceptible to infection with amastigotes of *L. major*, suggesting that the uptake of the parasites by *scid* mouse macrophages may be regulated by different receptor-ligand interactions.

In summary, both the Fc and CR3 receptors were shown to be involved in the phagocytosis of amastigotes. Differences in the entry of amastigotes obtained from the two sources were due to the different host components bound to the parasite surface at the time of isolation from the host and may reflect the extracellular molecules encountered by the parasite as it enters

from one macrophage to another during a natural infection. Other as yet unidentified extracellular matrix components are likely to be present on the parasite surface. The results of studies described in the next chapter were concerned with identifying molecules on the amastigote surface that may be involved in binding receptors on the host cell.

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Chapter VI: The Surface and Antigenic Structure of Amastigotes.

INTRODUCTION

Although the surface of promastigotes of *L. major* has been well characterized, little is known about the surface of amastigotes. This is primarily due to the difficulties in obtaining sufficient amounts of amastigote material from infected hosts. Recently, several methods of culturing amastigotes in vitro have appeared in the literature; however, the use of amastigotes obtained from in vitro cultivation has not been fully accepted. For example, *L. mexicana* amastigotes obtained from in vitro culture produce higher levels of LPG than amastigotes isolated from mouse lesions (T. Ilg, unpublished, cited by Winter *et al.*, 1994). Therefore, further research must be done to establish whether in vitro cultivated amastigotes truly represent parasites obtained from infected hosts.

The promastigote cultivated in vitro, differentiates from a non-infective (log phase) organism to an infective (stationary) stage (Sacks and Perkins, 1984; Da Silva and Sacks, 1987). The non-infective promastigote has a 7 nm coat, whereas the surface of the infective promastigote consists of a smooth, 17 nm thick coat, covering the entire surface and the flagellum. The amastigote surface, on the other hand, has a barely discernible surface coat (Pimenta *et al.*, 1991).

Several molecules have been identified on the surface of the promastigote and all are anchored by glycosyl-phosphatidylinositol anchors (GPI) on the outer leaflet of the parasite membrane. The major glycoprotein is the protease gp63. It is present at $5x10^5$ copies per cell and comprises 1% of the total protein content of promastigotes (Bouvier *et al.*, 1985). A family of minor, related proteins also are expressed: as a 90-92 kDa molecule in *L. major* (Murray *et al.*, 1989) and a 46 kDa molecule in *L. amazonensis* (Kahl and McMahon, 1987). Recently, a low molecular surface protein has been characterized from *L. donovani* promastigotes. It is an 11 kDa molecule, previously known as the LPG-associated protein (Jardim *et al.*, 1991) and now called the kinetoplastid membrane protein -11 (KMP-11) (Jardim *et al.*, 1995). This molecule is expressed at copy numbers of $1\text{-}2 \times 10^6$ molecules per promastigote. It is also present in the amastigote stage of *L. donovani* though

the amount and distribution of KMP-11 on the amastigote surface is not known.

LPG is the major glycolipid of the promastigote with 1-4x10^6 copies expressed per promastigote of *L. major* (Orlandi and Turco, 1987). The smaller molecular weight glycoinositol phospholipids (GIPL/s) are the major glycolipids on the amastigote surface. The GIPLs are expressed in promastigotes but are more abundant in the non-infective than the infective stages (Elhay *et al.*, 1988). There are approximately 1 x 10^7 copies of GIPLs on the surface of amastigotes and promastigotes of *L. donovani* (McConville and Blackwell, 1991), *L. mexicana* (Winter *et al.*, 1994) and 4 x 10^6 copies in amastigotes of *L. major* (Schneider *et al.*, 1993). It is estimated that there are sufficient numbers of GIPL's exposed on the surface to completely cover the amastigote; however, this has not been demonstrated. Amastigotes also possess a non GPI-anchored surface molecule that is a glycosphingolipid (GSL). The GSL accounts for 30% of the cells' glycolipids in *L. donovani*, and is host derived (McConville and Blackwell, 1991). The GSL is also found in *L. major* (Schneider *et al.*, 1993), *L. amazonensis* amastigotes (Barbiéri *et al.*, 1993; Straus *et al.*, 1993), and *L. mexicana* amastigotes where it is uniformly distributed over the surface (Winter *et al.*, 1994).

Using electron microscopy, it was shown that the major molecule in the coat of the infective promastigote is the glycolipid LPG (Pimenta *et al.*, 1991). Although gp63 is the major surface glycoprotein of promastigotes, Pimenta *et al.* (1991) demonstrated very little labeling of gp63 on the promastigote surface, presumably due to the LPG coat masking gp63. Interestingly, although gp63 is down-regulated in the amastigote stage (Schneider *et al.*, 1992), higher amounts of gp63 are detectable on the amastigote surface, using immunoelectron microscopy, than are detected on the promastigote stages (Pimenta *et al.*, 1991). LPG; however, is found in very low amounts on the amastigote surface.

The *scid* mouse was chosen as the source of amastigotes in my studies for three main reasons. First, the yield of amastigotes obtained from infected *scid* mice is ten fold higher than that obtained from BALB/c mice (as was shown in Chapter III). Secondly, the amastigotes from the *scid* mice have fewer host components bound to their surface. It was shown in Chapter V (Figure 5-2), that amastigotes from *scid* mice do not possess host immunoglobulins on their surface, whereas those obtained from BALB/c

mice are covered with parasite-specific immunoglobulin. Finally, using the *scid* mouse as the source of parasites reduces the contamination of the preparation with lymphocytes. BALB/c mice have high numbers of both T and B cells at the site of infection, that contribute to the swelling of the footpad itself (as shown in Chapter III).

To study the parasite ligands involved in binding macrophage receptors requires a knowledge of the surface molecules that are potentially involved in the amastigote-macrophage interaction at the cell surface. In this chapter of the thesis I describe the results of studies that examined the surface structure of the amastigote. The source of amastigotes and procedures used to yield relatively large quantities of material for the studies are also described. The surface of the *scid*-source amastigote was characterized using two different surface labeling techniques and finally a description is given of the antigenic structure of amastigotes (obtained from either *scid* mice or BALB/c mice) compared to promastigotes.

RESULTS

Source and isolation of amastigotes:

The technique used to isolate the amastigotes from the footpads of *scid* mice is described in Materials and Methods, Chapter II. Rehomogenizing the first pellet containing tissue debris and host cells increased the yield of amastigotes by 30%, with a total yield of approximately 1-2 x 10^9 amastigotes per mouse. NH_4Cl was effective in lysing the red blood cells without altering the viability of the amastigotes. This treatment was necessary because numerous red blood cells were contained within the amastigote cell suspension. Passage of the cells through a 27 gauge needle was used to assist in rupturing host cells that did not lyse during homogenization in the Ten Broek homogenizer and rapid vortexing of the amastigote suspension further aided in rupturing macrophages and freeing the amastigotes of host bound tissue. The amastigotes that were isolated using this technique were viable, as determined by FDA-EB staining, and capable of transforming to the promastigote stage in vitro. In addition, the amastigotes were free of cellular debris, and virtually free of host cells as the suspension consisted of greater than 99.9% amastigotes. An occasional intact host cell was observed when the final cell suspension was stained with FDA-EB and examined under the

microscope; however, there was never more than one host cell per hemocytometer grid that contained over 1,000 amastigotes. Occasional screening, throughout the course of the study, for the presence of antibody on the surface of the amastigotes using the ELISA technique revealed that the amastigote surface was free of antibody.

An alternative technique to lysis of red blood cells using NH_4Cl was incubation of the cell suspension in 0.01% SDS for 10 min. Both macrophages and red blood cells were lysed by this treatment; however the total yield of amastigotes was reduced by 55%. Therefore, NH_4Cl treatment was routinely employed in the preparation of amastigotes to be used for surface labeling and protein isolation.

To determine whether the amastigotes were free of macrophage membranes, the amastigotes were examined by transmission electron microscopy. It can be seen in Figure 6-1 that the amastigotes are free of the membrane from the host cell parasitophorous vacuole that surrounds the amastigote in vivo. In addition, the morphology of the amastigotes appeared normal.

Surface labeling:

Iodination:

The iodogen technique using Iodobeads® was employed to radiolabel live whole amastigotes. Initial screening of the level of counts per minute (CPMs) associated with amastigotes during the iodination procedure demonstrated that an 11 min exposure of the label and amastigotes to the catalytic beads resulted in maximum labeling of the surface. Free ^{125}I was removed by centrifugation and the level of ^{125}I in the supernatant was monitored. It was found that 6 rounds of centrifugation was sufficient to remove unbound ^{125}I, as the level of free ^{125}I did not decrease any further with continued centrifugation. The entire procedure was carried out on ice to minimize internalization of bound ^{125}I. The level of incorporation of label was reproducible since, in four different iodinations that were performed the level of CPM incorporated per 10^8 amastigotes ranged from 0.88×10^6 to 3.40×10^6, with a mean of $2.50 \pm 0.56 \times 10^6$ per 10^8 amastigotes.

After labeling with ^{125}I, the cells were stained with FDA-EB and the viability determined to be unaffected. In addition, amastigotes were capable of transforming to the promastigote stage when placed in culture medium at

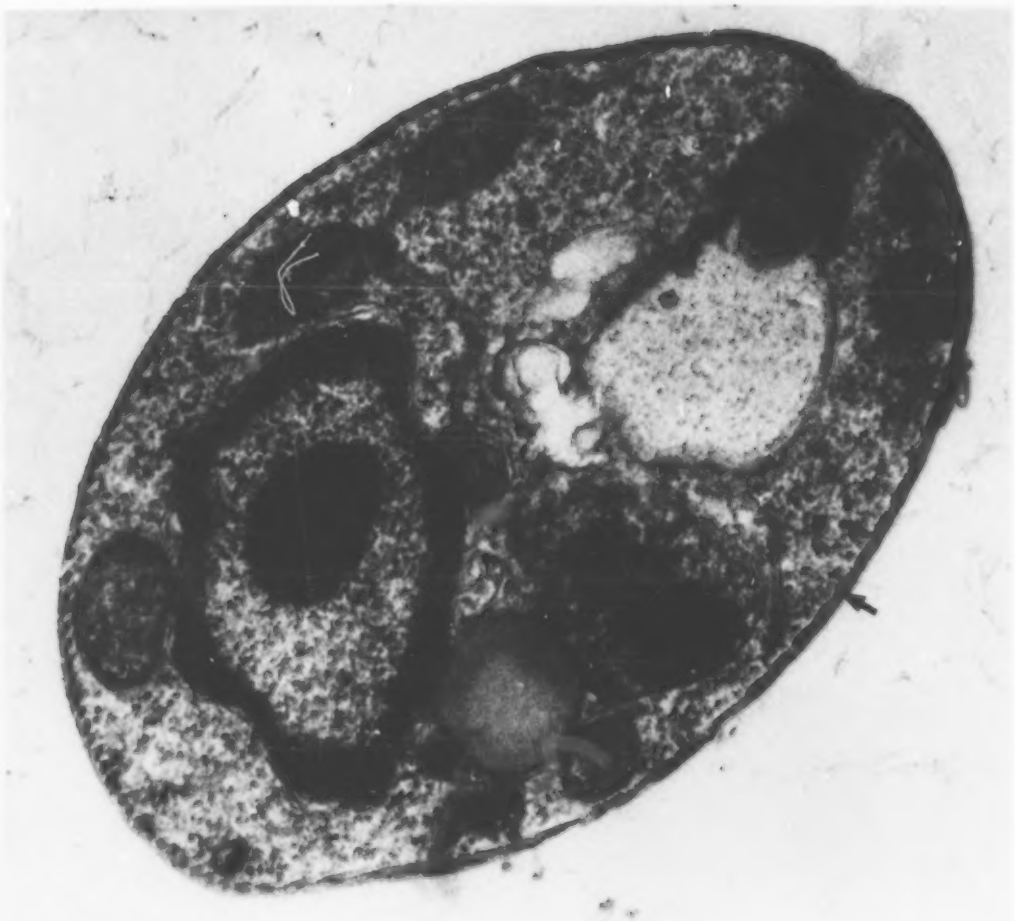

Figure 6-1: A transmission electron micrograph of a purified amastigote of *L. major* isolated from the footpads of *scid* mice. The arrow points to the outer membrane of the amastigote. The magnification was x 45,300.

RT. It was assumed, for two reasons, that the labeling was confined to the surface of the parasite: 1). the procedure did not affect the viability of amastigotes indicating that the membranes of the cells were unaltered; and 2). the procedure was performed on ice which prevented internalization of ^{125}I.

Repeated freeze-thawing was used to rupture the radioiodinated cells and the material fractionated by centrifugation. Table 6-1 shows the levels of radioactivity in different fractions following the procedures used to partially purify the membranes. Some of the radioactivity was lost in the first fraction; the pellet obtained from centrifugation at 3,000 x *g* to remove nuclear material and unlysed cells. The supernatant consisted of the membranes and cytoplasmic fractions of the cell and contained 70% of the total iodinated material. After centrifugation of the supernatant at 100,000 x *g* for 1 hr, 41% of the label was located in the pellet containing the membrane fraction and 28% was in the supernatant. This high amount of label in the 100,000 x *g* supernatant probably consisted mainly of free label that was released following freeze-thawing. When the soluble fraction was analyzed by SDS-PAGE no bands were observed. However, when the soluble fraction was acetone precipitated to concentrate the proteins, a 12 kDa band and a minor band at 43 kDa were observed.

Very few bands were obtained when the 100,000 x *g* pellet containing membranes was suspended in sample buffer, run on a 5-20% gradient gel using SDS-PAGE, and exposed to X-ray film (Figure 6-2). Only two major bands were visualized at 13.8 and 11.5 kDa, plus a large, very low molecular weight band ranging from 4-9 kDa (Figure 6-2, lanes 1 and 2). Seven minor bands of 28, 48, 72, 97, 120, 143, and 204 kDa were observed when larger amounts of starting material were loaded on the gels ($1x10^5$ CPM) and the X-ray films were left for longer exposure times, 72 hr (Figure 6-2, lanes 3 and 4). The presence or absence of β-mercaptoethanol did not alter the pattern.

Biotinylation:

To test whether the paucity of surface ^{125}I-labeled molecules observed on the autoradiographs was due to a limited number of surface exposed molecules or whether amastigotes possess few molecules bearing tyrosine or histidine residues available for binding to ^{125}I, the biotinylation method of surface labeling was performed. The biotinylation technique had no adverse affect on the live amastigote: cell viability was unaltered and biotinylated amastigotes transformed into promastigotes.

Table 6-1: Iodination of *L. major* amastigotes.

Amastigote Fraction	CPM/1x10^8 Cells	% of Initial
Intact Amastigote		
Trial 1	2.29 x 10^6	100%
Trial 2	1.80 x 10^6	
3,000 x *g* Supernatant		
Trial 1	1.40 x 10^6	61%
Trial 2	1.40 x 10^6	78%
100,000 x *g* Pellet		
Trial 1	0.80 x 10^6	35%
Trial 2	0.86 x 10^6	48%
100,000 x *g* Supernatant		
Trial 1	0.60 x 10^6	26%
Trial 2	0.54 x 10^6	30%

Amastigotes obtained from *scid* mice were iodinated using iodobeads. The amastigotes were ruptured by freeze-thawing and fractionated by centrifugation. CPM = counts per minute of ^{125}I measured in a gamma counter. Trial 1 and trial 2 represent the results from 2 separate iodinations.

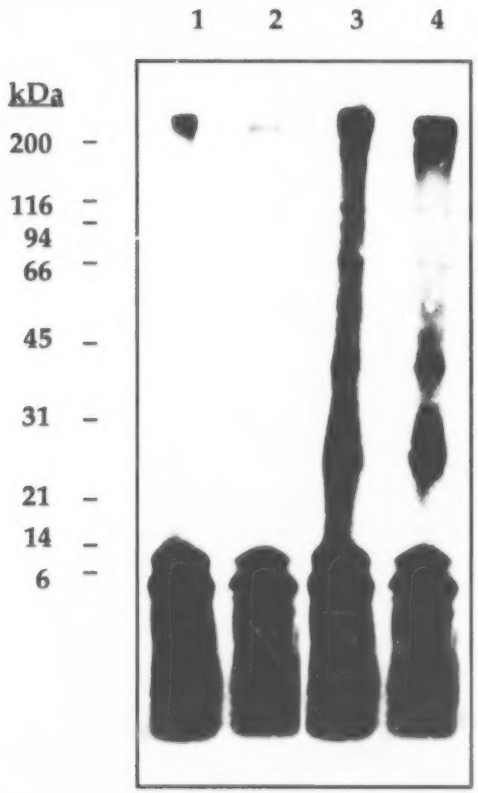

Figure 6-2: An autoradiograph of ^{125}I-labeled *scid*-source amastigotes of *L. major*. The membrane fraction of radiolabeled cells was run on a 5-20% gradient gel and exposed to X-ray film. The samples in lanes 3 and 4 were exposed for a longer period of time than in lanes 1 and 2 to observe the minor bands. The samples in lanes 1 and 3 were run under non-reducing conditions and samples in 2 and 4 were under reducing conditions. The migration of molecular weight standards (kDa) is indicated to the left of the autoradiograph.

Two major bands were labeled using the biotinylation technique (Figure 6-3, lanes 1 and 2). A very low molecular weight band of 13.7 kDa and a 66 kDa band that was not labeled using ^{125}I were detected. A number of minor bands were also present. The detection of biotin using avidin-alkaline phosphatase was specific because no bands were detected when non-biotinylated whole amastigotes were run on the gel and exposed to avidin-alkaline phosphatase (Figure 6-3. lanes 3 and 4). Many bands were observed (Figure 6-3, lanes 5 and 6) when a soluble fraction of amastigotes (obtained from the supernatant following 13,000 x *g* centrifugation of lysed amastigotes) was biotinylated and run on the gel. Therefore, there are numerous proteins within amastigotes that are capable of reacting with biotin. In addition, the conditions used for blotting, 0.22 µm nitrocellulose with a 2 hr transfer period then fixation in 0.2% glutaraldehyde, did not affect the reactivity of biotin to the blots. These conditions served to enhance the number and concentration of proteins transferred to nitrocellulose. Therefore, the scarcity of banding was not due to inefficiency of protein transfer or binding to nitrocellulose.

The banding pattern obtained using the two techniques is summarized in Table 6-2. More bands were detected using the biotinylation compared to iodination. A number of bands were detected using both techniques such as bands of 143 kDa and 48 kDa. In addition, several bands were unique to each technique: a 204 and 97 kDa band was detected only by iodination, whereas the 66 kDa band and several others were seen only using biotinylation.

Detergent solubilization of ^{125}I-labeled cells:

To isolate and identify the surface exposed membrane molecules, radioiodinated cells were solubilized in detergents. Four different detergents were tested: the zwitterionic detergent CHAPS and three non-ionic detergents, Triton X-114, Triton X-100 and N-octylglucoside. Following detergent solubilization and centrifugation at 100,000 x *g* the detergent soluble material in the supernatant was run on SDS-PAGE and the gel reacted against X-ray film. Only very faint bands could be detected. Since the material was too dilute to detect, the material was concentrated by precipitation in acetone and the precipitate was loaded onto the gels. Two major bands of 13.8 and 11.5 kDa and the low molecular weight smear were observed (Figure 6-4, lanes 1 and 2). Three minor bands were visualized with prolonged exposure to the X-ray film: 98, 47 and 31 kDa. Interestingly, identical results were obtained using all four detergents. The same bands were easily seen in the 100,000 x *g* pellet that

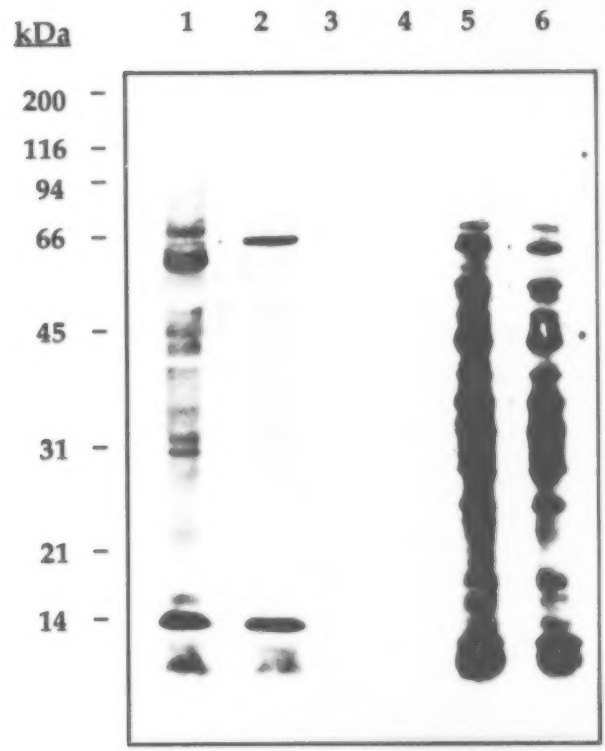

Figure 6-3: Avidin-alkaline phosphatase blots of biotinylated *scid*-source amastigotes of *L. major*. In lanes 1 and 2, live amastigotes were biotinylated then subjected to SDS-PAGE. Lanes 3 and 4 contain non-biotinylated amastigotes. Lanes 5 and 6 contain amastigotes that were lysed prior to biotinylation. The samples were run under non-reducing conditions (lanes 1, 3, and 5) or reducing conditions (lanes 2, 4, and 6). Migration of the molecular weight standards (kDa) is indicated to the left of the blot.

Table 6-2: A comparison of the molecular weights of surface labeled molecules of amastigotes using two different labeling techniques.

Iodination	Biotinylation
204	
143	142
120	123
97	
72	
	66
	57
	51
48	47
	32
28	29
	20
	16
13.8	**13.8**
11.5	11.8
9-4	

Live amastigotes, obtained from *scid* mice, were surface labeled using either ^{125}I or biotin. Labeled cells were solubilized in reducing sample buffer and run on a 5-20% gel. The numbers in bold represent major bands, all other bands were minor. Molecular weights were estimated from a standard curve based on the relative mobility's of proteins of known molecular weights.

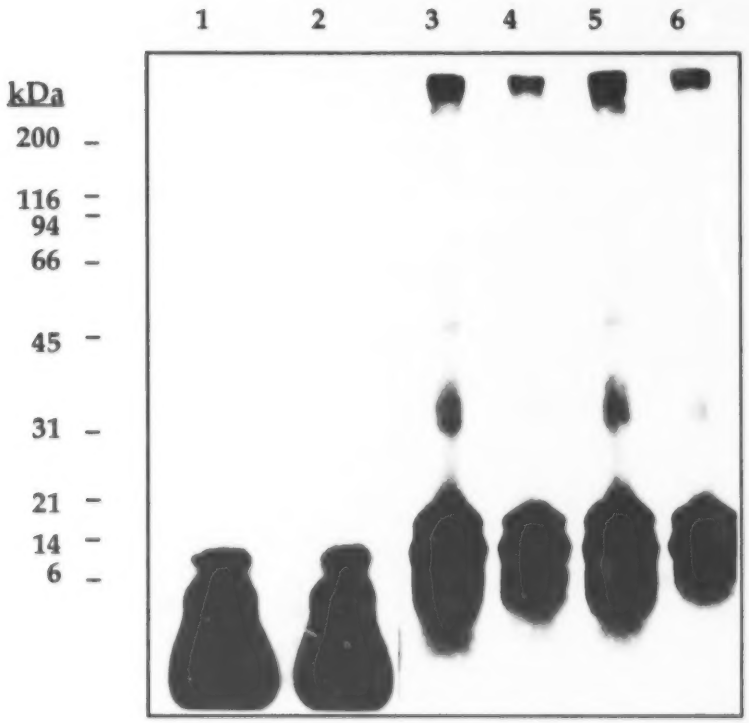

Figure 6-4: An autoradiograph of detergent solubilized and non-solubilized fractions of ^{125}I-labeled amastigotes obtained from *scid* mice. Lanes 1 and 2 contain 2% Triton X-100 detergent solubilized membrane proteins. The detergent insoluble material from the pellet following detergent treatment are in lanes 3 to 6: lane 3 = 1% Triton X-114; lane 4 = 2% Triton X-100; lane 5 = 2% CHAPS; lane 6 = 2% N-ocytl glucoside. Samples were run on a 5-20 % gradient gel in SDS-PAGE and exposed to X-ray film. The sample in lane 1 was under non-reducing conditions and lanes 2 to 6 were under reducing conditions. The migration of molecular weight standards (kDa) is indicated to the left of the autoradiograph.

contained the unsolubilized membrane proteins following detergent treatment, indicating that the detergents had not solubilized all the membrane proteins (Figure 6-4: lanes 3 to 6). There were no differences in the molecular weights of the bands or the amount of unsolubilized proteins when comparing the four detergents, therefore, the detergents had a similar ability to disrupt the parasite membranes.

Antigenic structure of amastigote:

Immunoblots:

Immunoblot analysis was used to study the antigenic structure of the parasite. Antisera against the BALB/c-source amastigotes and promastigotes recognized antigens from both stages. However, the promastigote antisera detected more promastigote antigens and more intensely than BALB/c-source amastigote antigens. Conversely, the BALB/c-source amastigote antisera reacted more intensely with BALB/c-source amastigote antigens and revealed a greater number of bands than on the promastigote antigen (Figure 6-5).

Antisera against amastigotes from BALB/c or *scid* mice recognized similar antigens, with the exception that the rabbit anti-BALB/c-source amastigote serum recognized an additional major 56.8 kDa molecule on BALB/c amastigotes (Figure 6-6). The 56.8 kDa molecule was not visualized when the rabbit anti-BALB/c-source amastigote serum was reacted with *scid* -source amastigote antigen, indicating that it is an antigen specific for amastigotes from BALB/c mice. The rabbit anti-BALB/c-source amastigote serum also recognized a major band of 50 kDa on promastigote antigen (Figure 6-5, left panel) that was not detected by the anti-*scid*-source amastigote serum (Figure 6-6, left panel).

A major feature of all the bands recognized by the rabbit antisera was that low molecular weight bands (less than 100 kDa) were detected in the amastigote antigen extracts obtained from *scid* mice, whereas low and high bands were seen against the amastigote antigen from BALB/c mice as well as the promastigote antigen. High molecular weight bands were detected in the *scid*-source amastigote antigen lanes when blotted against mouse antisera obtained from infected animals (Figure 6-7). Interestingly, the mouse antisera did not recognize bands lower than 13 kDa, whereas a common and strong feature of all blots against rabbit antisera were the bands at approximately 13.7, 11.5, 10 and the smear that ranged from the 11.5 or 10 kDa band down to

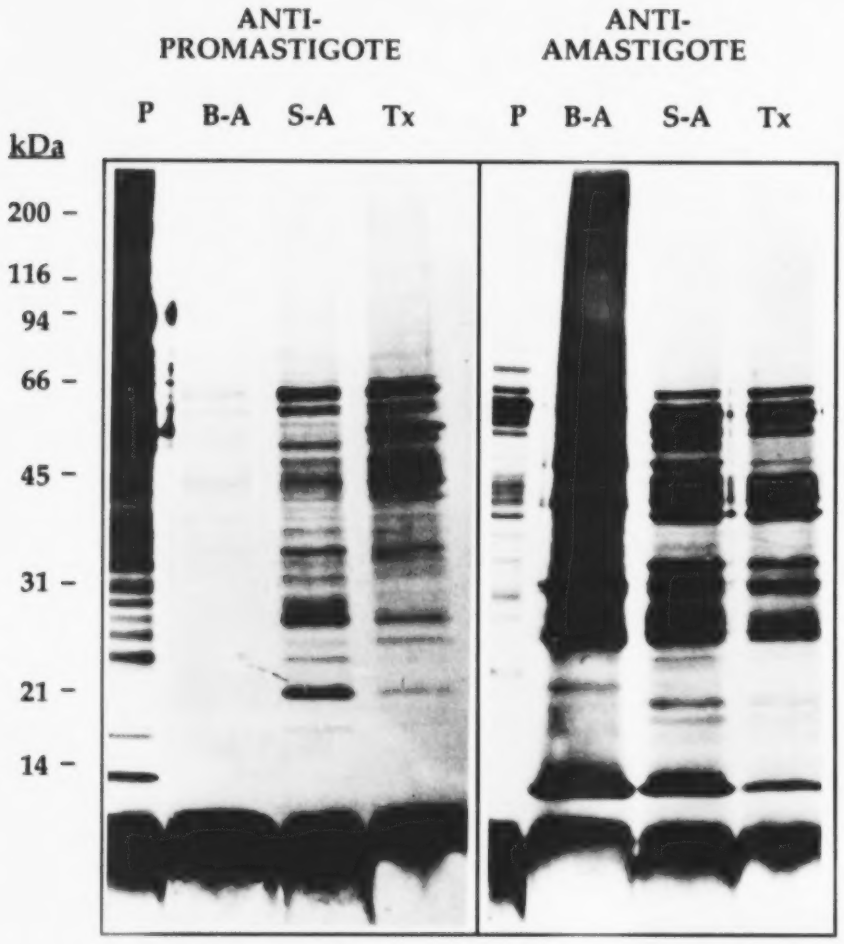

Figure 6-5: An immunoblot of *L. major* antigens using rabbit antisera raised against promastigotes or amastigotes. The antigens in the left panel were reacted against rabbit anti-promastigote serum. The antigens on the right panel were reacted against rabbit anti-amastigote serum. The antigen in each lane was subjected to reducing conditions and represent: P = promastigote; B-A = amastigotes from BALB/c mice; S-A = amastigotes from *scid* mice; Tx = Triton X-100 soluble molecules from *scid*-source amastigotes. The migration of molecular weight standards (kDa) is indicated to the left of the autoradiograph.

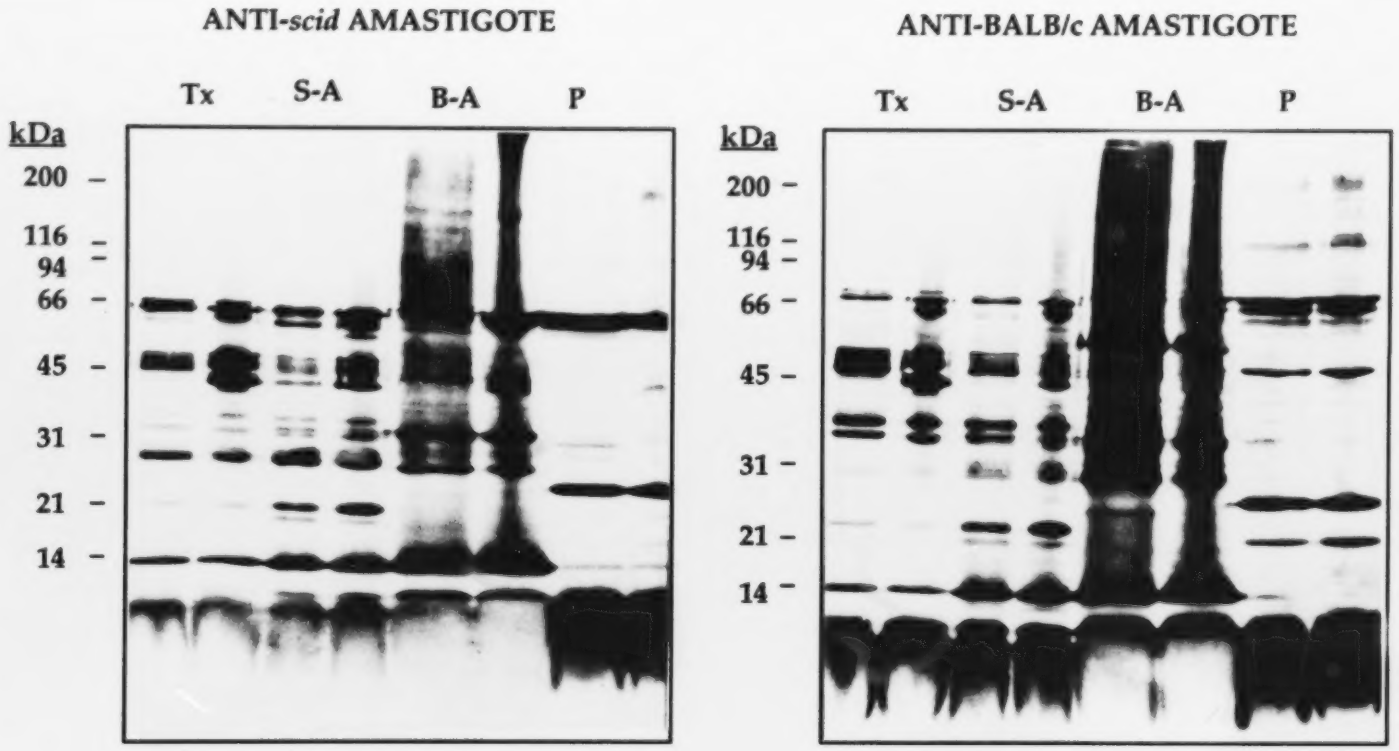

Figure 6-6: An immunoblot of *L. major* antigens using rabbit antisera against amastigotes obtained from *scid* or BALB/c mice. Antigens in the left panel were reacted against rabbit anti-*scid* source amastigote serum and the antigens on the right panel were reacted against rabbit anti-BALB/c source amastigote serum. See Figure 6-5 for details of the antigens in each lane. The migration of molecular weight standards (kDa) is indicated to the left of the autoradiograph.

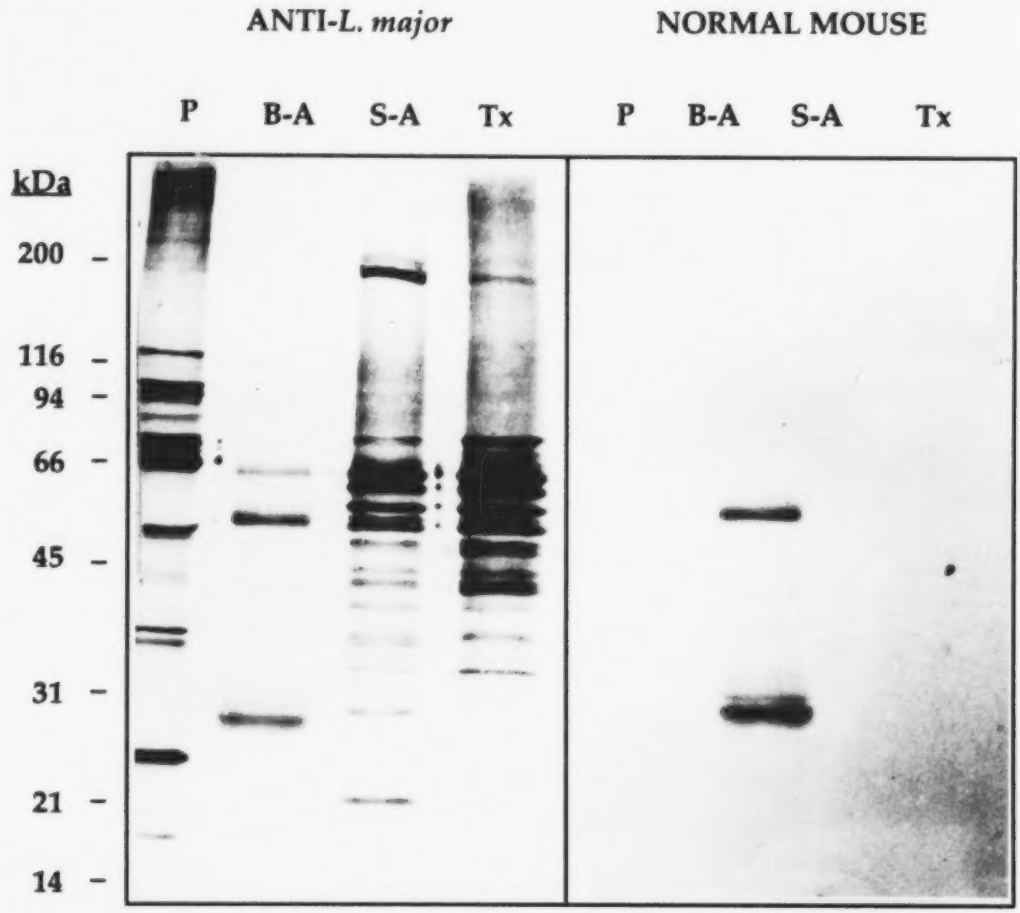

Figure 6-7: An immunoblot of *L. major* antigens reacted with serum obtained from BALB/c mice. The antigens in the left panel were reacted with sera from *L. major* infected mice and the antigens on the right were reacted with non-infected mouse serum. See Figure 6-5 for details of the antigens in each lane.

approximately 6 kDa. These very low molecular weight bands were not due to non-specific binding or an artifact near the dye front of the gel, because these bands were not detected by normal-rabbit or mouse sera, neither were they detected by the rabbit anti-serum made against promastigote gp63 (Figure 6-8). The 13.5 kDa band was a major component of both *scid*-source and BALB/c-source amastigotes that were reacted with rabbit anti-whole amastigote sera (against either *scid*-source or BALB/c-source amastigotes), whereas they were detected only faintly with rabbit anti-*scid*-source amastigote integral membrane (Figure 6-9) and not at all by the anti-promastigote serum. The 11.5 kDa band was detected on all antigen lanes by all the antisera. Whereas the 10.5 antigen was seen in *scid*-source amastigote and promastigote antigen preparations but not in lanes containing BALB/c amastigote antigen.

Antibody on the surface of the BALB/c-source amastigotes was detected by anti-mouse IgG-HRP. Two bands were seen when the BALB/c-source amastigote antigens were reacted against normal mouse serum (Figure 6-7, right panel). These bands, 60 and 29 kDa, correspond to the heavy and light chains of the antibody generated under reducing conditions. The bands were not detected in the other antigen lanes (Figure 6-7, right lane); however, they were a strong component of BALB/c amastigotes reacted with serum from *L. major* infected mice (Figure 6-7, right panel).

Tables 6-3 to 6-5 present a summary of the bands that were detected for each antigen, comparing the bands obtained when blotted against the six antisera. Table 6-3 represents *scid*-source amastigote antigen. Table 6-4 represents the BALB/c-source amastigote antigen blots and Table 6-5 the promastigote antigen blots. Very few differences were detected in blots comparing the *scid*-source antigen to the Triton X-100 detergent extract of *scid*-source amastigotes, therefore, only the whole *scid*-source amastigote antigen was represented (Table 6-3). One noticeable difference, besides differences in the intensity of bands, was a shift in a low molecular weight doublet detected using mouse anti-*L. major* serum. A 21 and 19 kDa doublet was detected using whole *scid*-source antigen, whereas a 23 to 21 kDa doublet was detected in the Triton X-100 extract. This shift of molecular weight maybe due to altered mobility of the proteins in the presence of Triton X-100.

A 73 kDa antigen was recognized in all parasite extracts and by all four antisera against whole parasites. The 73 kDa antigen was observed using amastigote antisera; however, the anti-promastigote serum recognized 2

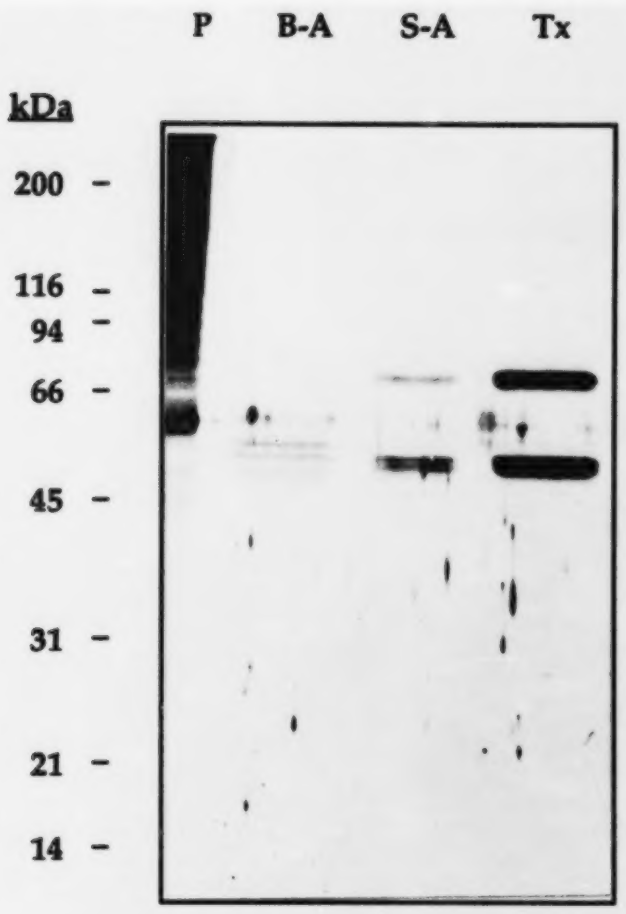

Figure 6-8: An immunoblot of *L. major* antigen using rabbit anti-promastigote-gp63 antiserum. See Figure 6-5 for details of the antigens in each lane. The migration of molecular weight standards (kDa) is indicated to the left of the autoradiograph.

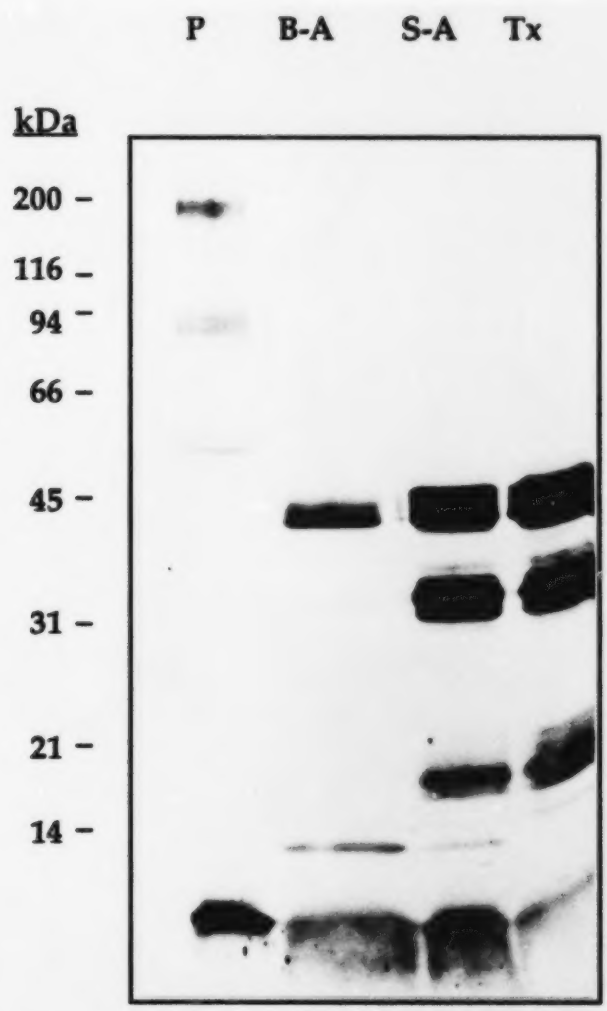

Figure 6-9: An immunoblot of *L. major* antigen using rabbit anti-*scid*-source amastigote integral membrane antiserum. See Figure 6-5 for details of the antigens in each lane. The migration of molecular weight standards (kDa) is indicated to the left of the autoradiograph.

Table 6-3: A summary of the molecular weights of the bands obtained when different anti-*Leishmania* sera were reacted against the *scid*-source amastigote antigen.

Anti-*Leishmania* sera					
R α *scid*-A	R α BALB/c-A	R α P	M α *L.major*	R α S-A I.M.	R α gp63
			156		
			83		**84**
		76			
73	73	70	**71**		
			68		
					59
54-46	**54-46**		**54**, 49	**54-43**	
35-32	35-32	36	36	**38-32**	
			31		
27	**27**	28			
			23		
20	**20**	20	**21**		
18.5	18.5		19		
				17	
			15.6		
13.7	**13.7**			13.5	
11.5	11.5	11.7			
10-6	10-6	10-6		10-6	

R= rabbit: M= mouse, A= amastigote; P= promastigote.

R α S-A I.M. = antibodies against the integral membrane molecules of *scid* amastigotes.

R α gp63 = antibodies against the glycoprotein gp63 from promastigotes.

Table 6-4: A summary of the molecular weights of the bands obtained when different anti-*Leishmania* sera were reacted against the BALB/c source amastigote antigen.

Anti-*Leishmania* sera					
R α *scid*-A	R α BALB/c-A	R α P	M α *L.major*	R α S-A I.M.	R α gp63
146	146	147	144		
134	134				
			80		
		76			
73	73	70	71		
			60		63-55
	57			54-43	
48	48				
32	**32**			36	
			29		
26	**26**				
			23		
13.6	**13.6**			**13.5**	
11.5-6	11.5-6	11.5-6		11.5-6	

R= rabbit: M= mouse, A= amastigote; P= promastigote.

R α S-A I.M. = antibodies against the integral membrane molecules of *scid* amastigotes.

R α gp63 = antibodies against the glycoprotein gp63 from promastigotes.

Table 6-5: A summary of the molecular weights of the bands obtained when different anti-*Leishmania* sera were reacted against promastigote antigen.

Anti-*Leishmania* antisera					
R α *scid*-A	R α BALB/c-A	R α P	M α *L.major*	R α S-A I.M.	R α gp63
174	174	**172**	**176**	**176**	
		112	**118**		
104	104	**103**	**103**	105	
			97		
		89			
73	**73**	73	**71**		
				68	
		63		63	**63**
52			54		
	50				
48	48				
			37		
31	32	35	35		
24	**24**	23	**23**		
20			19		
			17		
			15.6		
13.6	13.6	13.2		13.5	
11.5	11.5	11.7			
10-6	**10-6**	**10-6**		**10-6**	

R= rabbit: M= mouse, A= amastigote; P= promastigote.

R α S-A I.M. = antibodies against the integral membrane molecules of *scid* amastigotes.

R α gp63 = antibodies against the glycoprotein gp63 from promastigotes.

bands of 76 and 70 kDa in the amastigote antigen lanes, whereas it only recognized a 73 kDa band in the promastigote antigen lane.

Major components of *scid*-source amastigotes were the complex of bands at 54-46 kDa and from 35-32 kDa. These bands predominated when blots were reacted against the rabbit anti-*scid*-source amastigote integral membrane serum and were also strongly detected by all other antisera except for rabbit anti-gp63. A strong band of 20 kDa was detected by all rabbit anti-sera against whole parasites and by the mouse serum (Table 6-3).

A 147 kDa was specific to BALB/c amastigotes (Table 6-4). Fewer bands were seen when anti-promastigote serum was reacted against BALB/c amastigotes than with the *scid*-source amastigote antigen or promastigote antigen.

A 176 kDa promastigote specific antigen was detected using all antisera against whole parasites as well as by rabbit anti-*scid*-source amastigote integral membrane (Table 6-5). This antigen appeared as a minor band when reacted with the rabbit antisera against whole amastigotes, whereas it was a major band when detected by anti-promastigote serum, serum from *L. major* infected mice and even rabbit anti-*scid*-source amastigote integral membrane serum.

Interesting differences were seen in the banding pattern when the different antigens were blotted with rabbit anti-gp63 serum. The serum detected a strong 63 kDa band in promastigotes, as expected, as well as a smear of heavy molecular weight bands. The pattern in amastigotes was very different. Antigen from amastigotes from BALB/c mice revealed faint bands of 63-55 kDa, whereas the amastigote antigen from *scid* mice revealed two strong bands of 84 and 59 kDa. The Triton X-100 detergent extract of *scid*-source amastigotes reacted with greater intensity than whole *scid*-source amastigote antigen (Figure 6-8).

Opsonization of amastigotes with anti-parasite antibodies:

Enhanced entry of amastigotes from *scid* mice into macrophages in vitro was observed when the amastigotes were treated with the six antisera (Table 6-6), indicating that antibodies within the various sera recognize surface exposed epitopes on the amastigotes. Maximal enhancement of 250-300% in entry of amastigotes was observed at a 1:500 dilution of serum. Phagocytosis through the FcR was due to specific binding of the antibodies to

Table 6-6: The increase in the uptake of *scid*-source amastigotes opsonized with anti-*Leishmania* sera into macrophages in vitro.

SERUM	% INFECTION	INCREASE
Normal Rabbit	8 ± 0.7	
Rabbit-anti-*Giardia lamblia*	10 ± 1.7	25%
Rabbit-anti-C3	11 ± 2.3	38%
Rabbit- anti-*scid*-source amastigote	30 ± 1.5	210%
Rabbit-anti-BALB/c-source amastigote	31 ± 1.0	210%
Rabbit-anti-promastigote	29 ± 2.0	190%
Rabbit-anti-amastigote integral membrane	35 ± 0.5	240%
Normal Mouse	12 ± 0.6	
Mouse infected with *L. major*	47 ± 1.7	290%

Amastigotes were preincubated with sera (at a dilution of 1:500 for rabbit antisera and at 25 µg/ml for mouse sera) then exposed to macrophages for 1 hr at a ratio of 2 amastigotes : 1 macrophage. The data are expressed as the mean ± S.E.M. of percent infected macrophages. The percent increase was determine by comparing the percent infected macrophages with amastigotes treated with normal serum compared to immune serum. Each treatment was done in triplicate and the data is representative of at least 2 experiments per treatment.

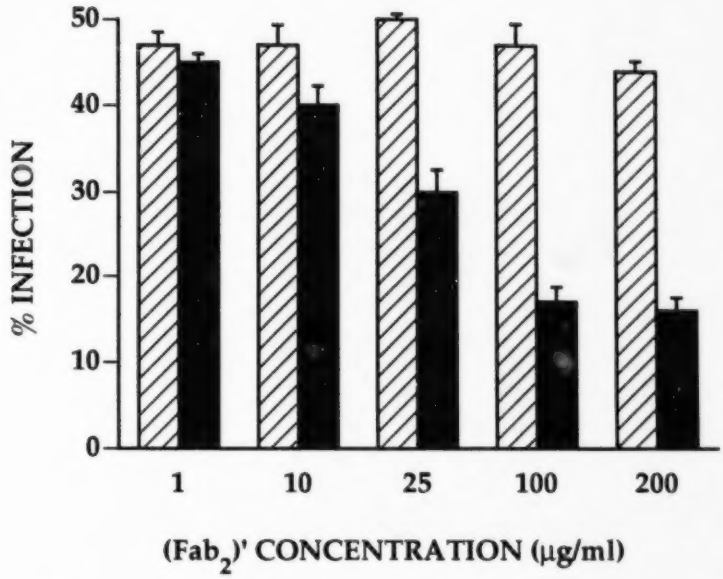

Figure 6-10: Blocking of phagocytosis of *scid*-source amastigotes in the presence of (Fab2)' fragments of rabbit anti-*scid* source amastigote integral membrane antiserum. The solid bars represent $(Fab_2)'$ fragments from anti-parasite serum and the vertical slashed bars represent normal $(Fab_2)'$ fragments. The data are presented as the mean ± S.E.M. and each treatment was done in triplicate.

amastigote surface molecules because treatment of amastigotes with normal rabbit or mouse serum did not enhance the uptake compared to unopsonized amastigotes. Two sources of immune serum, rabbit anti-*Giardia lamblia*, and rabbit anti-human C3, both containing high quantities of antibody, did not enhance the uptake of amastigotes into macrophages (Table 6-6). In addition, $(Fab_2)'$ fragments of antibodies, prepared by pepsin treatment of the rabbit anti-*scid*-source amastigote integral membrane serum, inhibited the uptake of amastigotes into BMDM compared to control $(Fab_2)'$ fragments (Figure 6-10). Blocking increased with increasing concentrations of the parasite-specific $(Fab_2)'$ fragments.

DISCUSSION

The *scid* mouse model proved to be an invaluable source of amastigotes for this study. The procedure I used to isolate the amastigotes resulted in high numbers of amastigotes free of cellular debris. During the phagocytosis of amastigotes by macrophages the amastigote is enveloped in the phagosome which fuses with lysosomes to form the parasitophorous vacuole. The amastigote remains within this vacuole throughout its life within the macrophage. The parasitophorous vacuole is associated with the amastigote surface when amastigotes are first isolated from mice (Chang and Dwyer, 1978) but this membrane is shed three to four hours after the amastigotes are isolated (Pimenta *et al.*, 1991). The procedure I used resulted in amastigotes free of the membrane, as seen using electron microscopy. A combination of time (several hours to complete the isolation), possibly the NH_4Cl treatment, as well as the fact that the amastigote suspension was vortexed at maximum speed before and after every manipulation, may all have contributed to removing the parasitophorous membrane surrounding the amastigote.

A few studies have been performed to examine the surface structure of amastigotes, using amastigotes obtained from BALB/c mice; however none have been done using the *scid*-source of amastigotes. Differences in the surface profiles and antigenic structure of amastigotes obtained from the two sources in vivo could potentially exist due to differences in binding of host molecules to the amastigote surface. As well, differential expression of antigens by the two sources of amastigotes may occur due to differences in the

environments to which they are exposed. The *scid*-source amastigotes are obtained from a severely immunocompromised host whereas BALB/c amastigotes come from an immunocompetant host.

In this study, radiolabeling of *scid* amastigotes revealed a few bands: a major triplet of 13.8 and 11.5 kDa, plus a large band ranging from 4-9 kDa, and seven minor bands of 28, 48, 72, 97, 120, 143, and 204 kDa. Comparing the surface labeling of *scid* amastigotes to published reports of labeling amastigotes from BALB/c mice showed that very few molecules were labeled and the major bands were low molecular weight molecules. Sadick and Raff (1985) radiolabeled amastigotes of *L. tropica* (= major) using two iodination techniques, the lactoperoxidase and iodogen methods, and obtained identical results. One major band of 9.5 kDa was detected when NP40 extracts of labeled amastigotes were subjected to SDS-PAGE. Two minor bands of 47 and 57 kDa and two faint bands of 24 and 35 kDa were also present. It is likely that the one band they detected running the samples on an 11% gel is a complex of the three low molecular weight bands, 13.5, 11.5 and 10 kDa, that I detected in *scid* amastigotes. A gradient gel of 5-20% may have been more effective in resolving the closely migrating, low molecular weight molecules.

Performing immunoprecipitation and 2-D analysis of radioiodinated parasites of *L. major*, Handman and Curtis (1982) reported six major bands of 50, 67, 68 and 80 kDa common to both the promastigote and amastigote stages. Two molecules of 43 and 94 kDa were unique to amastigotes. When ^{125}I labeled amastigotes were immunoprecipitated using a rabbit anti-promastigote serum, three major bands of 165, 90 and 66 kDa plus six minor bands were detected (Handman *et al.* 1984). They did not report the occurrence of molecules less than 20 kDa in either of their studies. It is possible that the low molecular weight bands migrated with the dye front under the conditions they used and thus were not reported, whereas low molecular weight molecules were a major feature of the autoradiographs obtained in my study and that of Sadick and Raff (1985).

All the studies, including mine, reported the presence of a 48-50 kDa molecule, whereas other molecules were seen in only some of the studies. For example, Sadick and Raff (1985) and the present study observed bands of 24-28 kDa. A 94-97 kDa band was observed in both the present study and that of Handman and Curtis (1982), whereas a 66-68 kDa band was a major feature solely in the studies of Handman and Curtis (1982) and Handman *et al.* (1984).

On the other hand, the minor bands of 72, 120, 143 and 204 kDa seen in my study were not reported by the others. These differences probably reflect the different methodologies that were employed.

No previous study has described the bands obtained when biotinylated amastigotes are subjected to SDS-PAGE. In the present study this procedure revealed several additional bands compared to the iodination procedure, bands of 66, 57, 51, 32, 20 and 16 kDa. This is probably due to the fact that the biotin binds to primary amines on lysine and free amino groups of other amino acids, which may be present in greater numbers than the tyrosine or histidine residues needed for ^{125}I binding (Billingsley *et al.*, 1987). Biotinylation of *scid* amastigotes revealed one of the major, low molecular weight molecules, 13.7 kDa, but also a major 66 kDa band, not seen when I performed the iodination procedure. A 60 kDa major band has previously been detected in amastigotes of *L. mexicana* under a slightly different technique: amastigotes that were metabolically labeled using ^{35}S-methionine, were then surface biotinylated, and the biotinylated surface molecules isolated using streptavidin-agarose (Medina-Acosta *et al.*, 1989). The major 66 kDa molecule of my study may be the same as the 60 kDa of Medina-Acosta *et al.* (1989); differences are likely due to differential glycosylation in the two species of *Leishmania*. Neither Sadick and Raff (1985) nor I detected the 60-66 kDa major band using iodination; however, Handman and Curtis (1982) detected a major 66 kDa band when radiolabeled amastigotes were immunoprecipitated using rabbit anti-promastigote serum.

The role of the protease gp63 in the biology of the amastigote stage is unclear. It has been detected on the surface of the amastigote by several different techniques including immunoelectron microscopy (Pimenta *et al.* ,1991) IFA (Frommel *et al.*, 1990) surface labeling (Medina-Acosta et al., 1989) and by immunoblotting (Frommel *et al.*, 1990). Schneider (*et al.*, 1992) reported that gp63 is downregulated in the amastigote stage. In my study I found that a major 66 kDa band was detected using biotinylation. Moreover, immunoblots reacting *scid* amastigote antigen with rabbit anti-gp63 serum detected 2 bands of 59 and 84 kDa, both were very strongly recognized in an antigen extract of Triton X-100 solubilized *scid* amastigotes. The rabbit anti-gp63 serum also enhanced the uptake of *scid* amastigotes into macrophages in vitro indicating the presence of surface exposed molecules. The profile I obtained in the immunoblot was slightly different from that obtained in

immunoblots using *L. mexicana* amastigotes (Frommel *et al.*, 1990). I detected the two bands of 59 and 85 kDa, whereas they observed one band, a higher molecular weight form than seen with promastigotes. This difference may be due to differential glycosylation between the two species of *Leishmania*.

Using the mouse antiserum numerous promastigote antigens were detected in immunoblots. This serum was obtained from mice infected with amastigotes of *L. major*. Since these animals had never been exposed to promastigotes, this demonstrates that there are numerous antigens and epitopes shared by the two stages of *Leishmania*.

Very low molecular weight bands were a strong component recognized by rabbit antisera. The characteristic smear ranging from approximately 11.5 or 10 kDa and extending to 6 kDa was also observed in the autoradiographs of radiolabeled membrane extracts and on an autoradiograph of the Triton X-100 detergent solubilized fraction of radiolabeled amastigotes, as well as on a silver stained gel of a Triton X-100 detergent solubilized extract of unlabeled amastigotes. Two studies using monoclonal antibodies against amastigotes have demonstrated the presence of very low molecular weight, surface located, amastigote-specific molecules. One mAb recognizes a 14 kDa doublet in *L. major* (Jaffe and Rachamin, 1989) and another mAb, specific to *L. panamensis* and *L. braziliensis*, recognizes a 12 kDa molecule (Eperon and McMahon-Pratt, 1989). The molecules recognized by the mAbs have not been characterized any further and I don't know if there is any relationship between these molecules and the low molecular weight molecules seen in my studies.

Handman *et al.* (1984) detected a 120 kDa promastigote-specific antigen using immunoblotting. I saw an approximately 114 to 118 kDa band on promastigotes only when anti-promastigote serum or mouse serum was used. Another promastigote specific antigen seen in my study was a 176 kDa molecule. It was not detected on blots of amastigote antigen; however, amastigote antisera detected this band in the promastigote stage. Interestingly, when I compared the profile obtained with promastigote antigen blotted against *L. major* infected BALB/c mouse serum to that obtained by Nguyen *et al.* (1984), I found that only a few bands were the same. Their profile using promastigote antigen was more similar to the profile I obtained with *scid* amastigotes. These differences may be due to the fact that I obtained the serum earlier in the course of infection, days 30-45 as opposed to day 77 in

their study. Another difference in the two studies is that I infected the BALB/c mice in the footpads whereas their mice were infected in the rump. These differences may have resulted in different antigenic profiles.

Antigens of *scid* amastigotes recognized by almost all sera were the 54 kDa and 36 kDa. All rabbit antisera recognized the 13.5 kDa molecule, and the 70 and 20 kDa molecules were recognized by rabbit antisera to whole parasites from both stages of the parasite. In my study the 54 and 36 kDa molecules were also visualized by biotinylation but not iodination. However, Sadick and Raff (1985) did detect 35 and 57 kDa bands on autoradiographs of iodinated amastigotes. A 55 kDa band has previously been reported in immunoblots of amastigotes of *L. major* obtained from BALB/c mice (Handman *et al.*, 1984). In my study, iodination of the cell surface revealed the 13.8 and 70 kDa molecules also seen in the immunoblots. Therefore, the molecules of 11.5, 13.8, 36, 54 and 70 all appeared to be located on the surface and were antigenic in nature.

Entry through the FcR during natural infection with *L. major* could involve binding of antibodies specific to a number of different epitopes, as was seen with the six anti-*Leishmania* sera. The anti-sera recognize different antigens, yet all enhanced entry of amastigotes into macrophages. Studies of this nature have not been previously performed using amastigotes because of the lack of Ig-free parasites. Chang (1981) demonstrated that opsonization of promastigotes, using rabbit anti-*Leishmania* serum, almost doubles the entry of promastigotes into macrophages in vitro.

In the past few years a number of studies have demonstrated that glycolipids are the predominant molecules on the surface of amastigotes (McConville and Blackwell, 1991; Schneider *et al.*, 1993; Winter *et al.*, 1994). From the studies reported in this chapter it can be seen that there are very few major surface exposed proteins in amastigotes of *L. major*. However, strong labeling of low molecular weight bands using both ^{125}I and biotin indicated that a low molecular weight protein may be an important component of the amastigote surface. In the following chapter I describe studies to isolate parasite surface molecules involved in binding of amastigotes to macrophage receptors.

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Chapter VII: Amastigote Ligands Involved in Entry of Amastigotes Into Macrophages.

INTRODUCTION

It is well established that entry of *Leishmania* into macrophages involves binding of specific parasite ligands to receptors on the macrophage surface inducing phagocytosis. Several receptors are involved in the phagocytosis of promastigotes by macrophages: the CR1 and CR3 (Blackwell *et al.*, 1985; Da Silva *et al.*, 1989), MFR (Basu *et al.*, 1991; Palatnik *et al.*, 1989), FnR (Rizvi *et al.*, 1988; Wyler *et al.*, 1985), AGE-R (Mosser *et al.*, 1987) and the FcR (Chang, 1981; Russell *et al.*, 1989). Both the CR3 and FcR are involved in the uptake of the amastigote stage of *L. major*, whereas the MFR is not involved (Guy and Belosevic, 1993; Chapter V).

Several promastigote ligands that bind to macrophage receptors have been identified in the past few years. Gp63 is the major glycoprotein that binds to macrophages. Binding of gp63 occurs directly to the CR3 (Wilson and Hardin, 1988; Russell and Wright, 1988) or through binding of complement component C3b to the gp63 molecule (Russell, 1987). Promastigote LPG binds to macrophage CR3 and CR4, either directly (Talamás Rohana *et al.*, 1990) or indirectly, through binding C3b (Puentes *et al.*, 1988).

LPG is also involved in entry of the amastigote stage of *L. major*. An 82% reduction in the attachment of *L. major* amastigotes to macrophages occurs in the presence of 1.42 μM amastigote LPG, or promastigote LPG. The backbone repeat units of LPG are the primary domain for binding to the macrophage (Kelleher *et al.*, 1995).

Another glycolipid, the glycosphinolipid (GSL) of *L. (L.) amazonensis*, causes a four fold decrease in uptake of amastigotes after a 4 hour exposure to macrophages in the presence of 4 μg of GSL. In addition, anti-GSL mAbs reduce the uptake of amastigotes into macrophages (Barbiéri *et al.*, 1993; Straus *et al.*, 1993).

Only one study to date has shown the involvement of a surface protein in the entry of amastigotes. The MFL or gp36 of promastigotes of *L. donovani* blocks the uptake of both the promastigote and amastigote stage into macrophages, 70% inhibition was seen in the presence of 100 μg/ml of MFL. The MFL on *L. donovani* amastigotes has not been isolated, therefore its

abundance in this stage of the parasite is not known. However, a rabbit anti-MFL serum recognizes the surface of live amastigotes in an IFA, although fluorescence of the promastigote stage was stronger than the amastigotes (Palatnik-de-Sousa *et al.*, 1993). This ligand is also employed in the entry of promastigotes of *L. mexicana amazonensis*, but promastigote blocking by MFL is species specific (Palatnik *et al.*, 1990). The role of the MFL in the entry of amastigotes of *L. major* is unknown, but its involvement is not likely since the MFL binds to the MFR on macrophages, a receptor that is not involved in amastigote entry (Guy and Belosevic, 1993).

In the previous chapter I identified the surface molecules of the amastigote stage of *L. major* and demonstrated that there are a few surface exposed proteins that may be involved in entry. In this chapter I discuss the results of experiments aimed to identify amastigote ligands that are involved in uptake into the host cell. First, I describe the procedures used to fractionate the cells. Then, I identify a molecule involved in blocking entry into macrophages. The data obtained in this portion of my research are preliminary and the physicochemical properties of the amastigote ligand have not been fully elucidated.

RESULTS

Fractionation procedure:

Amastigotes that were used to isolate proteins were obtained from *scid* mice, following the procedure outlined in Materials and Methods (Chapter III) and discussed in Chapter VI. Amastigotes that had been stored in PBS at -80^{o}C were warmed to 4^{o}C and a cocktail of protease inhibitors was added to prevent proteolysis of the molecules. The amastigotes were killed by freezing and thawing and further ruptured by sonication. Two crude fractions of amastigotes were prepared by centrifugation at 100,000 x *g* for 1 hr at 4^{o}C: the water soluble molecules in the supernatant, and the cell membranes and nuclear material contained within the pellet. The large plug of lipid formed at the top of the water soluble phase was carefully removed prior to collecting the supernatant. I routinely obtained approximately 130 mg of water soluble proteins, when a concentration of 1 x 10^{11} amastigotes was used as the starting material (Figure 7-1). The pellet containing the membranes was disrupted using a glass rod and suspended in a solution of PBS containing

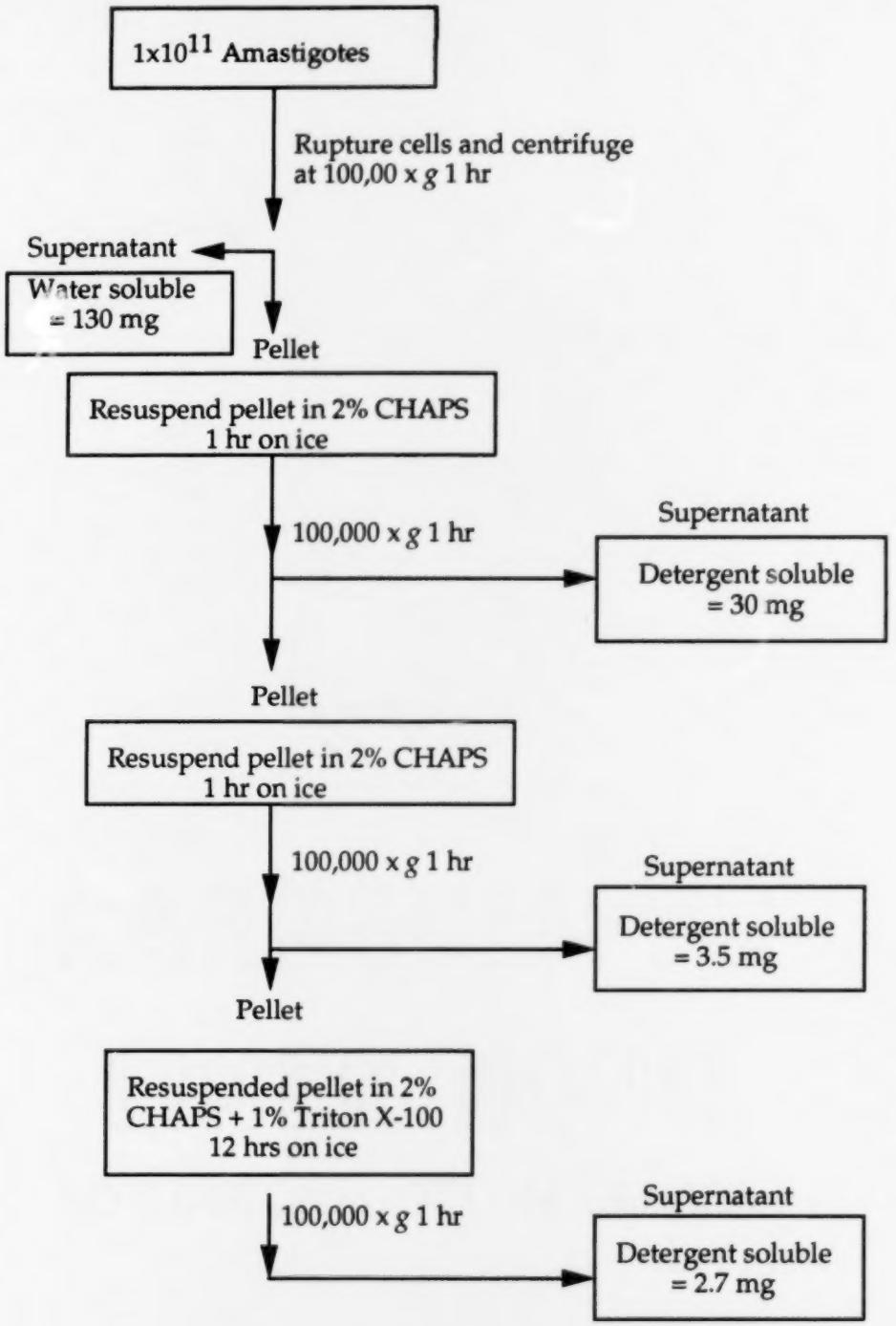
1x10^11 Amastigotes
Rupture cells and centrifuge at 100,00 x g 1 hr
Supernatant
Water soluble = 130 mg
Pellet
Resuspend pellet in 2% CHAPS 1 hr on ice
100,000 x g 1 hr
Supernatant
Detergent soluble = 30 mg
Pellet
Resuspend pellet in 2% CHAPS 1 hr on ice
100,000 x g 1 hr
Supernatant
Detergent soluble = 3.5 mg
Pellet
Resuspended pellet in 2% CHAPS + 1% Triton X-100 12 hrs on ice
100,000 x g 1 hr
Supernatant
Detergent soluble = 2.7 mg

Figure 7-1: A flowchart of the procedure used to isolate the water soluble and detergent soluble fractions of amastigotes. The concentrations of protein in the different fractions was determined using the Pierce protein assay.

2% detergent for 1 hr on ice. The 100,000 x *g* supernatant containing the detergent solubilized protein consisted of approximately 30 mg of protein. The same result was obtained whether I used 2% of Triton X-100, Chaps or N-ocytlglucoside, indicating that the three detergents had similar abilities to dissolve the membrane. In one experiment (Figure 7-1), the pellet containing the un-solubilized membranes from the first round of detergent treatment, was re-solubilized in fresh 2% Chaps for 1 hr at 4°C. After 100,000 x *g* centrifugation only 3.5 mg of protein was obtained. A third round of detergent treatment, involving re-suspension of the pellet in 2% Chaps, adding 1% Triton X-100, and incubation at 4°C for 12 hr resulted in only 2.7 mg of solubilized protein. Therefore, the majority of the proteins that could be solubilized in the detergents that were tested, were solubilized within 1 hr.

The two crude fractions of amastigotes were tested in blocking studies to determine whether they altered the uptake of amastigotes into macrophages in vitro. Blocking of amastigote entry was seen using both fractions when macrophages were pre-incubated in different concentrations of the fractions for 15 min. prior to addition of the amastigotes for 1 hr (Figure 7-2). The detergent solubilized fraction had greater blocking activity than the water soluble fraction. A 60% reduction in entry was obtained with 125 µg/ml of detergent soluble material as opposed to a 30% reduction in entry in the presence of the same concentration of the water soluble fraction. A high percent of detergent was present at these concentrations of detergent soluble molecules and the detergent alone reduced the entry of amastigotes into macrophages. However, the data are presented as the difference between the entry of amastigotes in the presence of the parasite ligand compared to entry in the presence of an equal concentration of detergent that was contained in the solution at that concentration of parasite ligand. Therefore, the reduction should be due to specific blocking of macrophage receptors by binding of the parasite ligand, but, a synergistic effect of detergent and parasite ligand on the macrophage cannot be discounted.

To study blocking of entry in vitro using membrane ligands requires a sufficient percent of detergent to maintain the ligands in solution, yet, low enough to prevent damaging the live cells. The effect of different concentrations of the three main detergents that were tested, on the viability of macrophages is shown in Figure 7-3. The macrophages were very sensitive to Triton X-100. Concentrations greater than 0.0025% Triton X-100 reduced

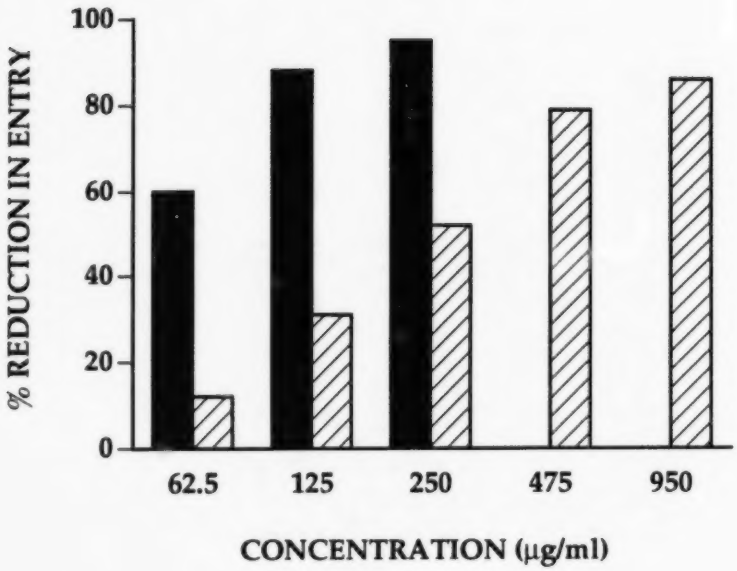

Figure 7-2: The reduction in the entry of *scid*-source amastigotes into macrophages in the presence of the water soluble and detergent soluble fractions of amastigotes. The slashed bars represent the water soluble fraction and the solid bars represent the detergent soluble fraction. The data are presented as the difference in the mean percent of infected macrophages in the ligand treated groups compared to the mean of controls. Each treatment was done in triplicate and the data are representative of three experiments.

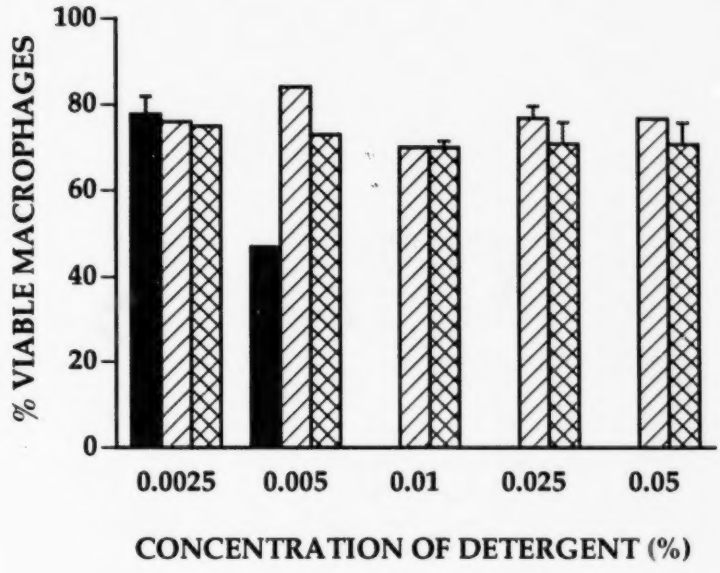

Figure 7-3: The viability of macrophages treated with different detergents. Three detergents were tested: Triton X-100 (solid bars), CHAPS (vertical slashed bars) and n-octylglucoside (hatched bars). The data are presented as the percent of viable cells in the treated groups compared to untreated macrophages. Viability was determined by microscopical examination of trypan blue treated cells. Each treatment was performed in triplicate and the data are presented as the mean ± the S.E.M.

macrophage viability. No macrophages were visible when viability staining was done after treatment with as low as 0.01% Triton X-100, whereas the macrophages were unaffected by concentrations of up to 0.05 % CHAPS or N-octylglucoside. The ability of macrophages to phagocytose amastigotes was reduced at detergent concentrations greater than 0.05% CHAPS and 0.025% N-octylglucoside (Figure 7-4). The percent reduction in entry was similar for the two detergents (Figure 7-4). To further study the effects of the membrane-derived, detergent solubilized ligands requires further purification of the suspension to reduce the concentration of detergent.

Iodacetamide, a protease inhibitor that I initially used in the isolation procedure, reduced the ability of macrophages to phagocytose amastigotes. The other protease inhibitors had no effect on phagocytosis so were maintained in the isolation buffer (Figure 7-5). The FPLC profile of the water soluble fraction was similar when the samples were isolated in the presence or absence of iodacetamide, indicating that the other protease inhibitors were sufficient to prevent proteolysis. The action of iodacetamide, to inactivate thiol proteases, was probably performed by antipain and leupeptin which have the same function.

Purification of a blocking ligand:

I concentrated on purifying a blocking molecule(s) from the water soluble fraction of the amastigote extract. Thirteen major peaks were observed when this extract was run on gel filtration chromatography, most of very low molecular weights. Peak 1 eluted with the void volume, and peaks 2 through 6 eluted at 199, 45.7, 7.6, 5.1, and 3.2 kDa. Peaks 7 through 13 were below 2 kDa and an extremely small molecular weight peak was routinely obtained after approximately 2 column volumes (Figure 7-6). The profile did not alter noticeably when the sample was filtered through a 0.22 µm filter or when the sample was stored at -80°C prior to use. Upon thawing, a precipitate was observed in the sample; however, the protein concentration and FPLC profile did not change when the precipitate was removed by centrifugation at 13,000 x *g*.

The fractions obtained from the FPLC run were tested in a blocking assay. No blocking was observed of amastigote entry into macrophages <u>in vitro</u>, when the blocking assay was performed on the proteins eluted in the peaks after loading the column with the maximum volume that contained

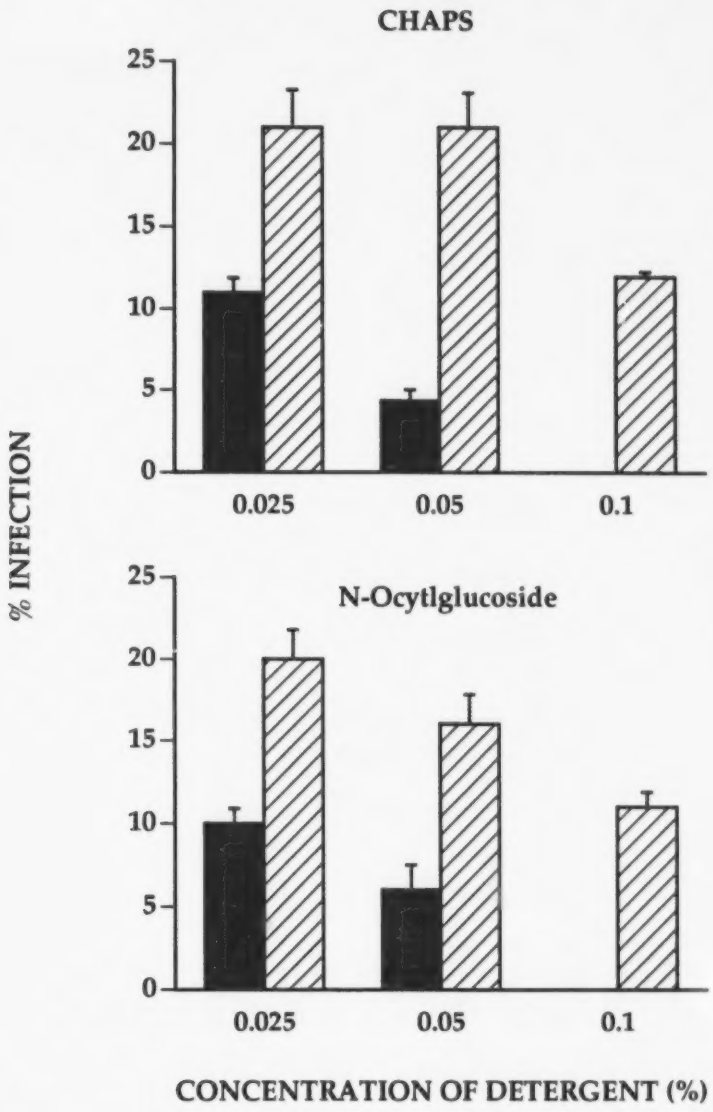

Figure 7-4: A comparison of the phagocytosis of amastigotes of *L. major* in the presence of the detergent soluble amastigote fraction compared with detergent alone. The solid bars represent the detergent solubilized membrane protein obtained from *scid*-source amastigotes and the hatched bars represent the detergent controls. The concentration of protein at the different percentages of detergent was 45 µg/ml at 0.025%, 90 µg/ml at 0.05%, and 175 µg/ml at 0.01%. The data are presented as the mean ± S.E.M. of 3 replicates per treatment and are representative of 2 to 3 experiments.

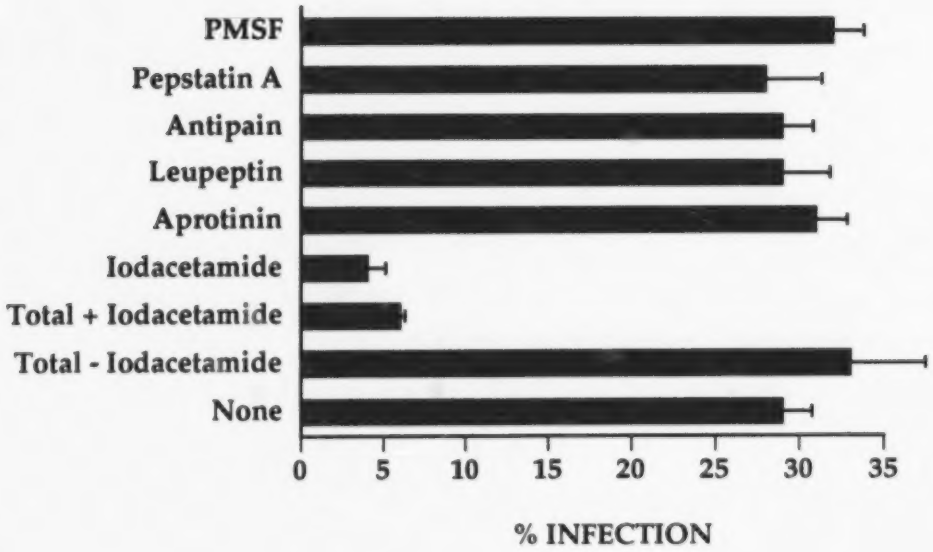

Figure 7-5: The effect of protease inhibitors on the ability of macrophages to phagocytose *scid*-source amastigotes. Macrophages were pretreated with protease inhibitors for 15 min. prior to addition of the amastigotes. The concentration of the protease inhibitors was : 1 μg/ml leupeptin, antipain, and pepstatin A; 5 μg/ml of aprotinin; 1 mM PMSF and 2.5 mM iodacetamide. The data were presented as the mean ± the S.E.M. Each treatment was performed in triplicate.

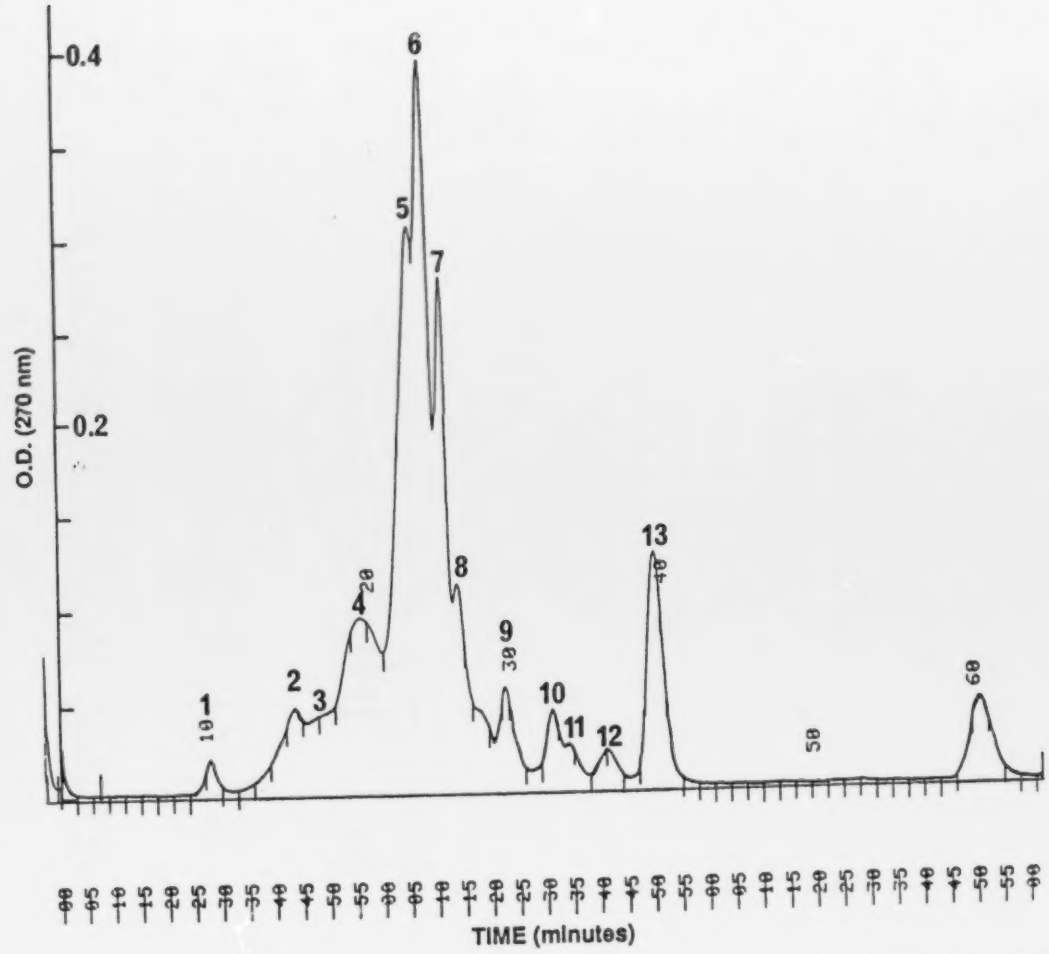

Figure 7-6: A chromatogram of the water soluble fraction of amastigotes using gel filtration chromatography. PBS pH 7.2 was used as the running buffer at a flow rate of 0.3 ml/min. The proteins were detected at 280 nm.

1.4 mg of the water soluble fraction of amastigotes. This was probably due to the low concentrations of protein within the peaks. To increase the protein concentration, successive samples were run on the FPLC and the eluted peaks were pooled. The sample volume in the peaks was concentrated by lyophylization, then dialyzed against PBS to remove the high salt concentration. The protein concentration following these procedures was still low, proteins may have been lost through the dialysis tubing or precipitated out of solution. However, when material from peaks 2 through 6 were pre incubated with macrophages in vitro, a 20% reduction in uptake of amastigotes was observed in the presence of peak 6 (Figure 7-7). The fractions from peaks 1 and 7 through 13 were not tested due to extremely low protein concentrations. The material from peak 6 eluted from the FPLC column as a 3 kDa molecule, estimated from a standard curve based on the elution times of molecular weight standards.

An alternative method of concentrating the starting material was followed, using filtration membranes and centrifugation. The water soluble fraction of amastigotes was centrifuged through a 25 kDa MW cutoff cone, the eluate was collected then centrifuged through a 3 kDa membrane and the fraction retained on the filter was kept for further analysis. The FPLC profile of this concentrated fraction consisted of 3 major peaks and several smaller, very low molecular weight peaks (Figure 7-8a). The molecular weights of these proteins were 13.5, 9.0, and 5.6 kDa. When the material retained on the 3 kDa membrane was run on a 5-20% SDS-PAGE gel and silver-stained, three main bands were observed corresponding to molecular weights of 17, 12.2 and 9.9 kDa (Figure 7-8b). Pre incubating macrophages with material from the 3 peaks obtained from the FPLC revealed blocking activity in the central peak, which corresponds to peak 6 in the elution profile of the entire water soluble fraction. A reduction of 29% in the entry of *scid* amastigotes into BMDM was seen at a concentration of 50 µg/ml (Table 7-1).

I have not succeeded in determining the purity of the material contained in peak 6 or in elucidating the molecular weight of the molecule(s) using SDS-PAGE. The molecule(s) in peak 6 was detected at an O.D. 280 on the fraction detector during the FPLC run, and was also detected using the Pierce protein assay but was not detected with the Bio Rad method of protein estimation. Three bands were readily detected when 10 µg of the 3 kDa retained material was run on the 5-20% gel (Figure 7-8b). Unexpectedly, no

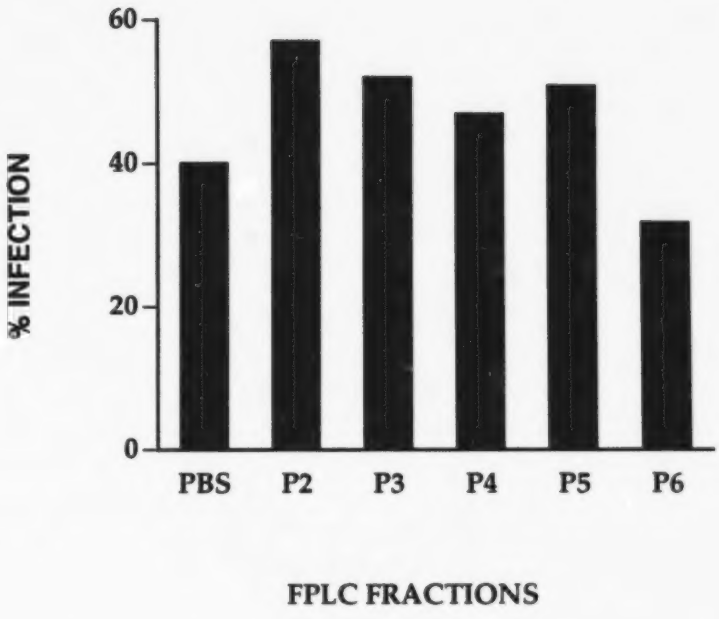

Figure 7-7: Blocking phagocytosis of amastigotes of *L. major* by protein(s) in peak 2 to 6. The data are presented as the percent of infected macrophages. The PBS control was performed in triplicate and is presented as the mean. Peaks 2 to 6 were single trials due to the limited amount of material.

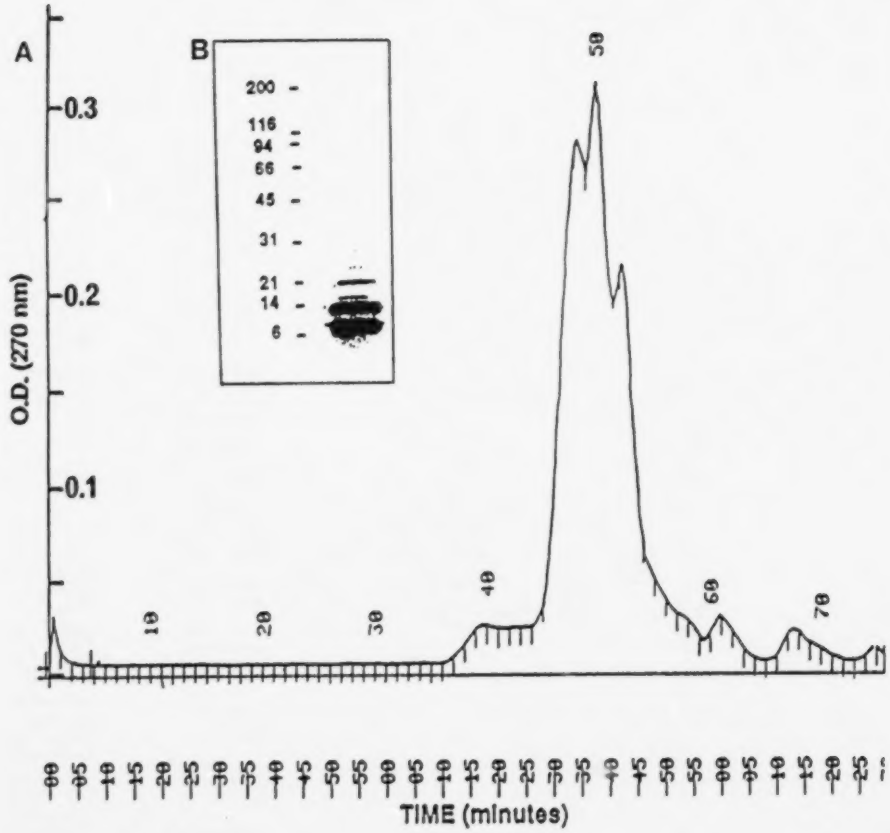

Figure 7-8: A chromatogram and an SDS-PAGE gel of the concentrated fraction of the water soluble molecule. A) The concentrated fraction was obtained by size exculsion of the water soluble amastigote fraction. B) A silver-stained 5-20% gel containing the concentrated fraction of the water soluble amastigotes. The migration of molecular weight standards is shown at the left hand side.

Table 7-1: Blocking of amastigote entry into macrophages by proteins in the major peaks from FPLC gel filtration of the size exclusion fraction of water soluble molecules isolated from amastigotes.

FRACTION OF WATER SOLUBLE EXTRACT FROM AMASTIGOTES	PERCENT INFECTION	PERCENT REDUCTION
PBS	21 ± 0.3	
Whole [a]	11 ± 1.2	50%
P5 [b]	24 ± 1.5	
P6 [c]	15 ± 1.5	29%
P7 [c]	22 ± 2.3	

The protein concentrations of parasite ligands were: a). 435 µg/tube; b). 25µg/tube; c). 50 µg/tube. The ratio of amastigotes to BMDM was 5:1. The data are presented as the mean ± S.E.M. of 3 replicates per group and the data are representative of 2 experiments with similar results.

bands were visualized when 3 µg of the concentrated proteins in peak 5 and 6 were loaded on the gels and silver-stained. The molecules in these peaks were not heat-labile or sensitive to reducing agents since the absence of β-mercaptoethanol in the sample buffer plus lack of heat treatment at 100°C did not produce bands. Copper staining the gel revealed a 21 kDa protein in the lane containing 8 µg of P5 but no band was seen in the lane containing 7 µg of P6 (Figure 7-9).

No bands were evident in immunoblots when the material from peaks 5 and 6 were reacted against rabbit anti-*scid* source amastigote serum or rabbit anti-*scid* source amastigote integral membrane serum. The crude fraction of the 3 kDa retained material did show bands; however, different bands were observed when reacted against the two different antisera. A major band of 29 kDa plus minor bands of 13.7, 14.8, and 79 kDa were visualized with the rabbit anti-*scid* source amastigote serum, whereas a strong band of 34 kDa and a minor 32 kDa band were visualized with the rabbit anti-*scid* source amastigote integral membrane serum (Figure 7-10). A rabbit anti-promastigote serum recognized a faint band of 29 kDa. No bands were seen when the antigen was reacted against normal rabbit serum.

The fractions in P5 and P6 were reacted with mAbs specific for an 11 kDa protein, the Kinetoplastid Membrane Protein-11 (KMP-11), recently identified in all trypanosomes and from *L. donovani*. A combination of two mAbs that recognize different epitopes on KMP-11 (McNeely *et al.*, 1990) were tested. The mAbs reacted against purified KMP-11 protein (Figure 7-11 lane 3), whereas no bands were detected in lanes containing 3 µg of either P5 or P6. KMP-11 forms polymers when isolated from the membrane, therefore the higher molecular weight bands (20 to 50 kDa), in lane 3 of Figure 7-11, are polymers of KMP-11. Reaction of the mAbs against amastigote antigen in an ELISA gave similar results. A high O.D. was observed when the mAbs were reacted with purified KMP-11, but not with the proteins contained in P5 or P6 (Figure 7-12). MAb L-98 produced a small response against the water soluble fraction of the *scid*-source amastigotes. Changing the concentration of antigen did not alter the results. The concentrations of amastigote antigens that were tested were: 0.1, 0.5, 1, 10, 50 and 100 µg for the water soluble, detergent soluble and 3 kDa retained material; and 0.3, 1 and 3 µg for P5 and P6.

The KMP-11 antigen was reacted against different anti-sera to *L. major* amastigotes to determine whether any of the anti-sera recognized the protein.

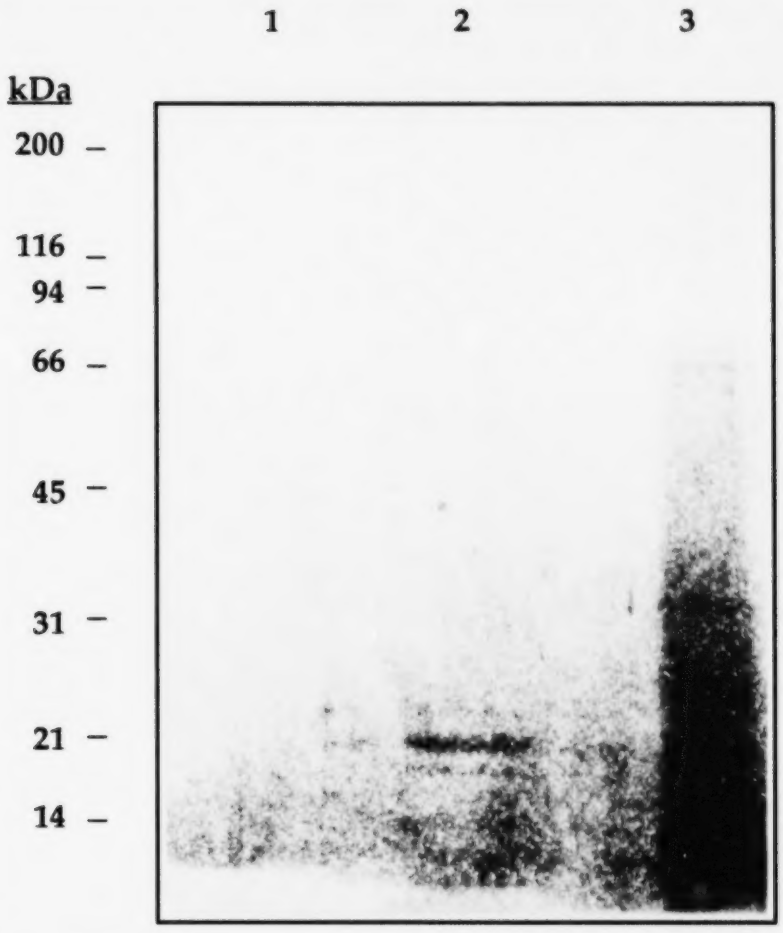

Figure 7-9: A copper-stained gel containing proteins in peaks 5 and 6 from FPLC purified material. The samples were run under reducing conditions on a 5-20% gradient gel. Lane 1 = peak 5; lane 2 = peak 6; lane 3 =KMP-11. Migration of the molecular weight standards (kDa) is indicated to the left of the gel.

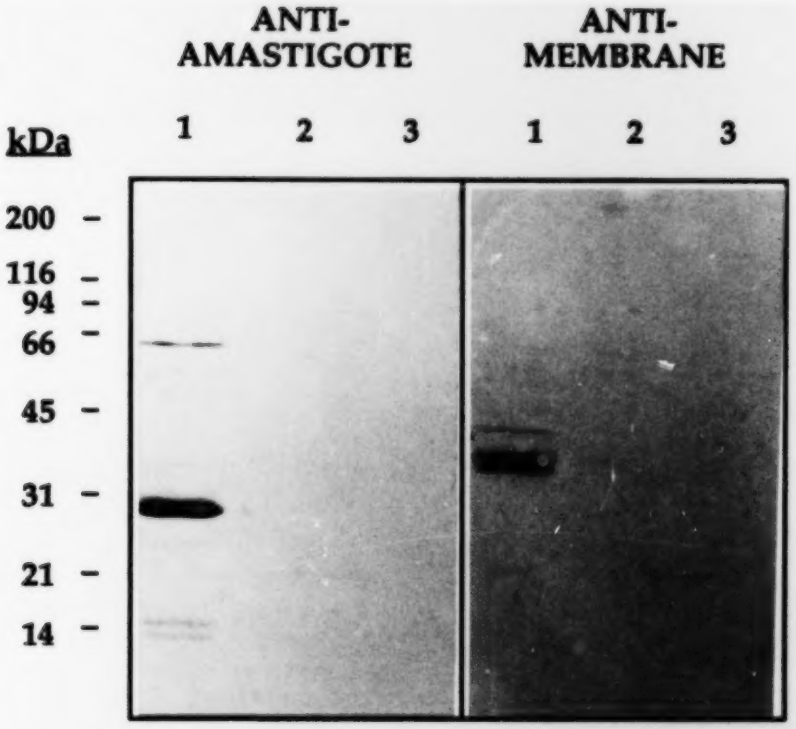

Figure 7-10: An immunoblot using rabbit anti-*L. major* antiserum to detect the proteins in peaks 5 and 6 from FPLC purified material. Lane 1 = water soluble concentrate; lane 2 = peak 5; lane 3= peak 6. The antigens were run under reducing conditions. Antigens in the left panel were reacted against rabbit anti-whole amastigote antiserum. and on the right were reacted against rabbit anti-integral membrane antiserum. Migration of the molecular weight standards (kDa) is indicated to the left of the blot.

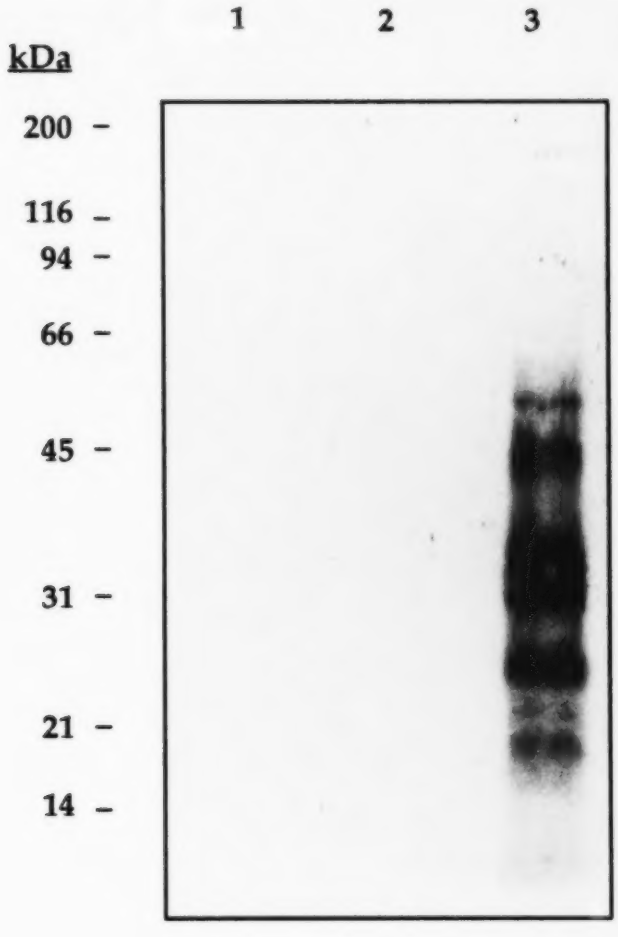

Figure 7-11: An immunoblot using anti-KMP-11 mAbs to detect the proteins in peaks 5 and 6 from FPLC purified material. Lane 1 = water soluble concentrate; lane 2 = peak 5; lane 3= peak 6. The antigens were run under reducing conditions.

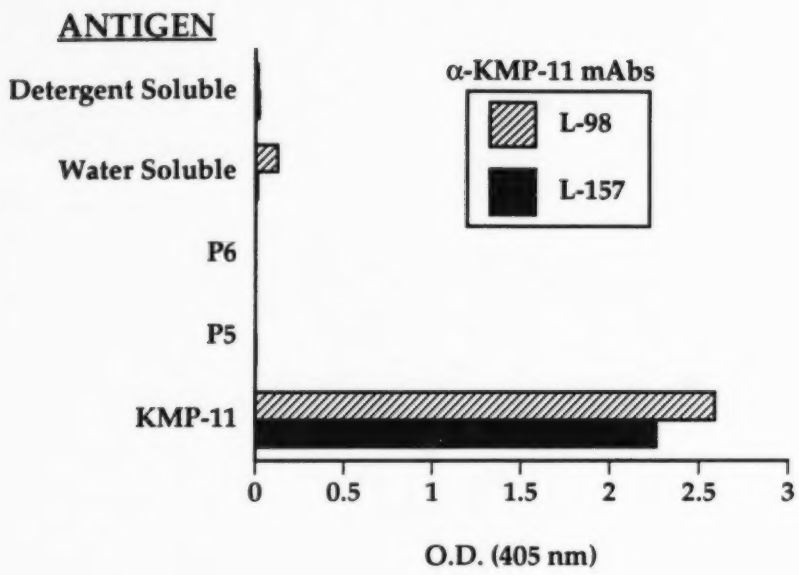

Figure 7-12: An ELISA using anti-KMP-11 mAbs to detect the proteins in peaks 5 and 6 from FPLC purified material. The concentrations of antigen were: 5 µg/well of purified KMP-11; and 10 µg/well of water and detergent soluble amastigotes; and 3µg/well of P5 and P6. The data are presented as the mean of 2 replicates per sample and are representative of 2 experiments.

The strongest recognition was from the serum obtained from a mouse infected with *L. major*; however, this response was not as strong as the KMP-11-specific mAbs (Figure 7-13). A very small response was observed with the rabbit anti-whole *scid*-source amastigote serum and no response was seen with the rabbit anti-amastigote integral membrane serum.

Indirect evidence suggests that the material in peak 6 may be derived from the surface membrane of amastigotes. An ELISA reacting the different fractions obtained from an FPLC run of the whole water soluble fraction of amastigotes revealed that several fractions were recognized by immune sera from rabbits. Peaks 2, 4 and 6, showed binding activity with the antiserum against whole *scid*-source amastigotes (Figure 7-14). The rabbit anti-*scid* source amastigote integral membrane serum had one major area of binding, around peak 6, with reactivity tailing into peaks 7 and 8 (Figure 7-14). Therefore, the peak containing blocking activity was strongly recognized by immune serum against membrane proteins.

Interestingly, a radiolabeled 13 kDa molecule was seen in SDS-PAGE when the water soluble extract from *scid*-source amastigotes was precipitated using acetone and the precipitate run on a gel (Figure 7-15). Only low amounts of this molecule were present as it required precipitation to visualize the protein. In addition to the 13 kDa molecule, a faint band of 43 kDa was also observed. These may both represent molecules shed from the surface by the amastigotes or molecules cleaved during the isolation procedure. A 13 kDa molecule was also a major component of the membrane fraction when detergent solubilized membranes were run on SDS-PAGE and the gel was silver-stained (Figure 7-16).

DISCUSSION

A limiting factor in elucidating the molecular interactions of the amastigote with its host cell has been the difficulty in obtaining sufficient quantities of the surface exposed molecules to study. Not only is the promastigote more easily attainable, due to in vitro cultivation techniques, but due to its larger size, more protein can be isolated per individual promastigote compared with amastigote.

Both the water soluble and detergent soluble fractions of the amastigotes contained parasite ligands that bind macrophage receptors, since

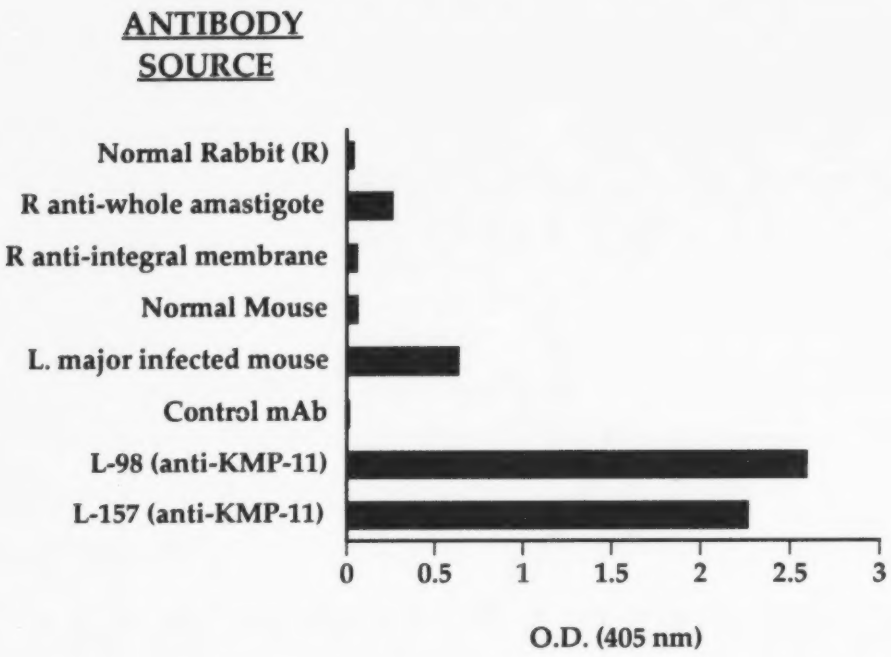

Figure 7-13: An ELISA using different antisera to detect purified KMP-11. The concentration of KMP-11 in each well was 5 μg. The data is presented as the mean of duplicate samples per treatment and is representative of 2 experiments.

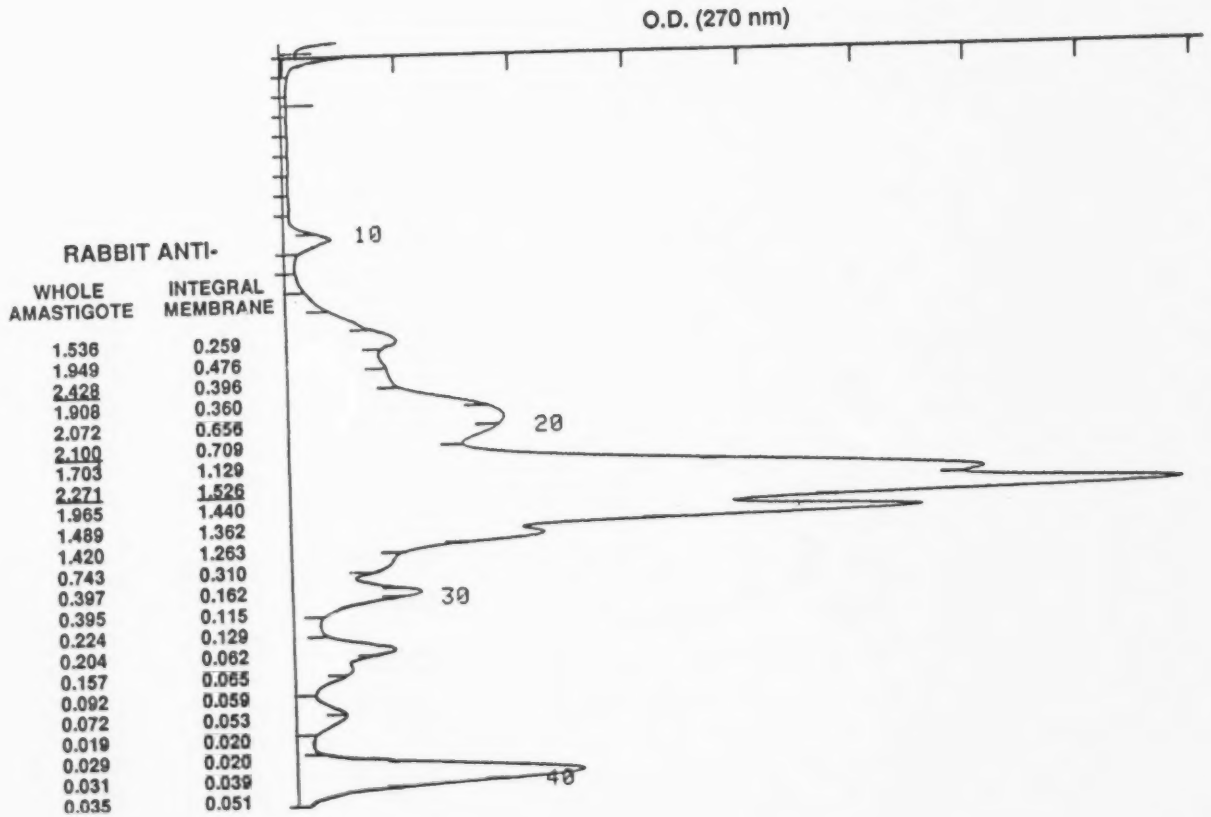

Figure 7-14: An ELISA using rabbit anti-*L. major* sera to detect the proteins in the peaks from FPLC purified material. To the right is shown the FPLC profile of the water soluble fraction of amastigotes. To the left is shown the optical density (O.D. 405 nm) of the different FPLC fractions reacted in an ELISA against either rabbit anti-*scid* source amastigote antiserum (left side) or rabbit anti-*scid* source amastigote integral membrane antiserum (right side).

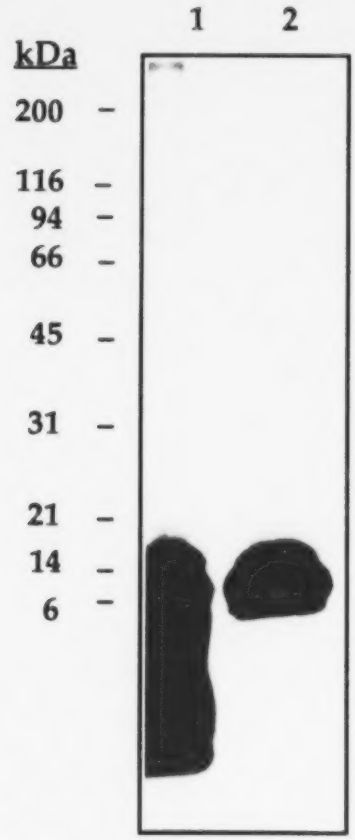

Figure 7-15: An autoradiograph of the water soluble and membrane fractions of ^{125}I-labeled amastigotes. Lane 1 contains the pellet following 100,000 x *g* of ruptured amastigotes. Lane 2 contains the acetone precipitate of the water soluble fraction of amastigotes. Both samples were subjected to reducing conditions. Migration of the molecular weight standards (kDa) is indicated to the left of the blot.

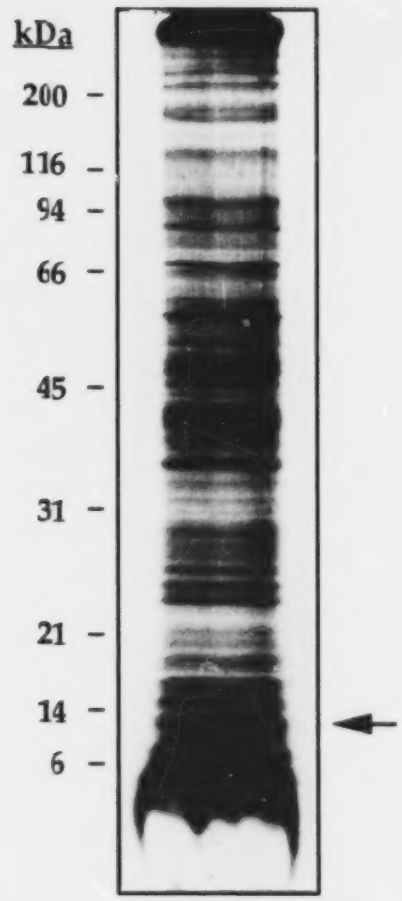

Figure 7-16: A silver-stained gel containing the Triton X-100 soluble membrane fraction from *scid*-source amastigotes. The sample was run under reducing conditions on a 5-20% gradient gel. The arrow points to a 13.5 kDa band. Migration of the molecular weight standards (kDa) is indicated to the left of the blot.

both fractions blocked the uptake of amastigotes into macrophages in vitro. Interestingly, comparing the solubilization of the amastigotes with the three detergents, TX-100, CHAPS and N-ocytlglucoside produced similar results. First, they solubilized a similar amount of protein from the membranes of amastigotes. Second, the same MW proteins were solubilized as observed in the autoradiographs of detergent solubilized ^{125}I labeled amastigotes (see Chapter VI). Finally, the CHAPS and N-octylglucoside solubilized extracts of the parasite blocked the entry of amastigotes into BMDM in vitro to a similar degree.

In my study, a small molecular weight molecule reduced the entry of amastigotes into macrophages by 30%. This level of blocking was over 66% of the entire blocking activity detected in the water soluble fraction from the amastigote, therefore the majority of blocking was attributable to molecule(s) contained within this peak. The molecular weight of this molecule as determined by gel filtration chromatography varied depending on the sample that was run on the column. It eluted either as a 9 kDa or 3 kDa molecule, eluting faster when the fraction that was obtained by size exclusion was run on the column and slower when the entire fraction of water soluble molecules was applied to the column. It is difficult to explain why I was unable to detect the molecule in peak 6 using SDS-PAGE. Three strong bands were detected using silver stain in the size exclusion fraction containing molecules between 25 and 3 kDa. Possibly these three bands do not correspond to the three major peaks obtained when this fraction was separated using gel chromatography. Other molecules not detected by silver stain were contained within this fraction since major bands of 32, 34 and 79 kDa were detected in the when the material was subjected to the immunoblot technique. The molecule(s) in peak 6 are antigenic since they were detected by immune sera in the ELISA. Interestingly, copper staining revealed a 21 kDa band in peak 5 not seen by silver staining or immunoblotting. It would appear that SDS-PAGE and immunoblot techniques alter the molecule(s) within peak 6 rendering them unrecognizable to antibodies and incapable of being stained.

There are several low molecular weight antigens on the amastigote membrane that have been identified. KMP-11, previously known as the LPG-associated protein is expressed on both the amastigotes and promastigotes of *L. donovani*. It is present at 1-2 x 10^6 copies on promastigotes. KMP-11 is a common antigen of all *Leishmania* and is also found in several species of

trypanosomes, detected using immunoblot with KMP-11 specific mAbs (Tolson *et al.*, 1994). MAbs to KMP-11 did not react with the blocking molecule(s) in my study in either the ELISA or immunoblot, thus it appears that the blocking molecule is not KMP-11. The location and abundance of KMP-11 in amastigotes is unknown. To date only a single study has briefly examined the molecule in the amastigote stage: KMP-11 isolated from amastigotes of *L. donovani* gave a positive reaction with mAb L98 (an α-KMP-11 mAb) in an ELISA (Jardim *et al.*, 1995). Although KMP-11 is present in all trypanosomes and can be detected with the mAbs L98 and L157, the protein isolated from *L. donovani* and *Trypanosoma brucei* markedly differ in their amino acid structure (Stebeck *et al.*, 1995). In addition, there is differential expression of KMP-11 in different life cycle stages: the blood stream forms of *T. congolense* express much lower levels than the procyclic culture forms, the epimastigotes and metacyclics. Thus, KMP-11 may be an important molecule in the insect vector and less so in the vertebrate host.

A major 12 kDa surface antigen, as well as minor bands of 260, 130 and 75-85 kDa have been identified using amastigote specific mAbs. This antigen was identified in *L. panamensis* and *L. braziliensis* but not in *L. infantum*, *L. amazonensis* or *L. pifanoi* (Eperon and McMahon-Pratt, 1989). It is not known whether these mAbs recognize KMP-11.

There are four reasons that suggest that the blocking molecule(s) in this study may be a membrane bound form that was released from the amastigote surface. 1). A 13 kDa molecule was observed in the supernatant containing water soluble molecules, from radiolabeled amastigotes. 2). Low molecular weight molecules were the major components of the amastigote surface, evidenced by surface iodination and biotinylation, shown in Chapter VI. 3). The low molecular weight molecules were also a major component of the detergent solubilized membranes. 4). The molecule(s) contained in peak 6 was strongly recognized by rabbit anti-serum against the integral membrane of *scid*-source amastigotes.

A number of molecules are known to be shed or released from the membranes of eukaryotic cells following proteolytic cleavage. Soluble forms of the FcγRII and III, the CR2, the intercellular adhesion molecule -1 (ICAM-1) and decay accelerating factor (DAF) are released from the membrane of mammalian leukocytes following enzymatic cleavage (reviewed by Bazil, 1995). In malaria, the merozoite surface protein-1 (MSP-1) on merozoites

undergoes several rounds of cleavage. In the second step the 42 kDa fragment of MSP-1 is cleaved into a 33 and 19 kDa fragments, the MSP-1 19 kDa fragment remains on the cell surface (reviewed by Breton and Da Silva, 1993).The function of which may be to expose parasite motifs to binding by host receptors.

Alternatively, several studies have shown the release of surface molecules during the disruption of the cell membrane. The MFL of *L. donovani* is obtained in the supernatant following 48,000 x *g* centrifugation of cells that are ruptured by freezing and thawing then heated for 15 min. at 100°C (Palatnik, *et al.*, 1989). Some of the malaria gp76 molecule is released during osmotic rupturing of the merozoites (Braun Breton, *et al.*, 1986).

Several *Leishmania* molecules are shed or secreted from the cells. LPG, also known as the excreted factor, is released as two forms, an intact form (King *et al., 1987*) and a hydrophilic form called phosphoglycan (Slutzky *et al.*, 1979). The acid phosphatases of *Leishmania*, 100-130 kDa molecules, are secreted from the parasite (Lovelace *et al.*, 1986). A soluble form of surface derived gp63 has also been reported. The soluble and membrane forms of gp63 have similar electrophoretic mobilities and protease activity (Bouvier *et al.*, 1985; Etges *et al.*, 1986). Eperon and McMahon-Pratt (1989) reported that their mAbs against a 12 kDa molecule detect fluorescence outside of the amastigote in IFA, indicating that the molecule is secreted into the medium. Secreted molecules of GIPL have been reported for the promastigote stage, these secreted molecules are rapidly degraded at 37°C (Elhay *et al.*, 1988). It seems unlikely that the low molecular weight blocking molecule in my study is a water soluble form of GIPL. The isolation procedure I used involved freezing and thawing, a period of approximately three hours at RT during fractionation on the FPLC and a 1 hr and 15 min. incubation at 37°C during blocking of macrophages. The combination of these procedures would likely denature the molecule if it were a soluble form of a GIPL.

Lipids have been implicated in the binding of amastigotes to macrophages; however, their role in phagocytosis has not been established. LPG inhibits the binding of *L. major* amastigotes to macrophages, but, the authors did not report whether the LPG also inhibits the entry of the amastigote in addition to preventing attachment (Kelleher *et al.*, 1995). Moreover, very little LPG is expressed on the amastigote surface, less than 10^4

copies of LPG are present in *L. major* amastigotes (Schneider *et al.*, 1993). It is unknown whether this level is sufficient to induce phagocytosis.

It has been observed that common structures are present in different surface molecules of the parasite. For example, the phosphorylated saccharide portion of LPG is also a carbohydrate component of a secreted acid phosphatase (Bates *et al.*, 1990; Jaffe *et al.*, 1990). Portions of LPG are also homologous to the GIPLs that are abundant on the amastigote surface; however, the GIPLs caused very little reduction in binding, only 28% reduction in the presence of 400 μM GIPLs (Kelleher *et al.*, 1995).
A mAb to GSL reduced the uptake of amastigotes into macrophages; however, this mAb not only recognizes GSL but also recognizes gp63 on promastigotes, it was not shown whether gp63 on amastigotes is also recognized (Barbiéri *et al.*, 1993).

In summary, a low molecular weight molecule(s) was purified from the water soluble fraction obtained from *scid*-amastigotes. This molecule(s) blocked the entry of *scid*-source amastigotes into BMDM by 30%. Over 60% of the total blocking activity of the water soluble material was contained in this fraction. Further studies are needed to determine the purity of peak 6 and to characterize the chemical nature of the blocking molecule. In addition, it remains to be determined whether this molecule(s) is a major component of the membrane surface.

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Chapter VIII: General Discussion:

To date, very few comprehensive studies have been conducted on the uptake of amastigotes of *Leishmania* into macrophages, despite the importance of this stage in the disease. Blackwell *et al.* (1985) examined the use of three receptors, the CR3, MFR and FcR in the uptake of amastigotes of *L. donovani;* however, this species of *Leishmania* causes visceral leishmaniasis and preferentially infects the liver and splenic macrophages. *L. major*, the cause of cutaneous leishmaniasis, preferentially infects inflammatory macrophages. Since these parasites are in different environments, and the different macrophage populations express different levels of phagocytic receptors, it is likely that different mechanisms are involved in the entry of these two species of *Leishmania*. There are also differences in the surface composition of the amastigotes, for example, *L. major* amastigotes express 6×10^4 copies of LPG on their surface (Moody *et al.*, 1993), whereas *L. donovani* express 10 fold less LPG (McConville and Blackwell, 1991). Therefore, the mechanisms of entry in other species of *Leishmania* cannot be assumed to be involved in *L. major*.

Other studies have briefly examined amastigotes in relation to promastigotes, but these studies were not comprehensive. For example, Wyler *et al.* (1985) studied the role of fibronectin in the binding of promastigotes and amastigotes to macrophages. Blank *et al.* (1993) demonstrated that the CR3 is used by amastigotes of *L. major* to enter epidermal Langerhan cells. A recent study was one of the first to identify an amastigote ligand, LPG, involved in amastigote binding to macrophages (Kelleher *et al.*, 1995); however, this is not an amastigote-specific molecule and was first identified on promastigotes (King *et al.*, 1987). Most research using amastigotes has been done to understand the mechanism of activation of macrophages for intracellular killing (Buchmüller and Mauel, 1979; Nacy *et al.*, 1981; Haidaris and Bonventre, 1981; Belosevic *et al.*, 1988), and to identify the killing mechanisms mediated by oxygen radicals (Murray, 1982; Channon and Blackwell, 1985, Scott *et al.*, 1985) and nitric oxide (NO) (Green *et al.*, 1990, Liew *et al.*, 1990; Mauël *et al.*, 1991).

There is still a large gap in our knowledge concerning the entry of amastigotes into macrophages. Thus, my research focused on the interactions of the amastigote stage of *L. major* with its host cell, the macrophage. My

working hypothesis was that the two stages of *Leishmania*, the promastigote and amastigote, differ in the receptors used to enter the macrophage. This is because these parasites encounter different macrophage populations in the mammalian host. The promastigotes are injected into the tissue and are probably phagocytosed by resident dermal macrophages (Locksley *et al*, 1988). The amastigotes preferentially infect immature macrophages that have been recruited to the lesion site (Fortier *et al.*, 1982; Davies *et al.*, 1988). Another difference between the entry of the two stages of *L. major* is in the environment where uptake into macrophages occurs. The promastigotes are in an acute inflammatory environment, whereas the amastigotes are in a chronic inflammatory environment. Additionally, molecules in the saliva of the sand fly enhance the infectivity of promastigotes in mice, possibly mediated by increased phagocytosis of the parasite (Titus and Ribeiro, 1988). For these reasons, I hypothesized that different mechanisms are involved in the phagocytosis of amastigotes compared to promastigotes.

The *scid* mice proved invaluable for my studies. They yielded a new source of amastigotes that were free of immunoglobulin, thereby making possible the study of the use of the FcR and providing a 'cleaner' parasite source for surface characterization. In addition, they demonstrated the importance of host proteins in the interaction of the parasite and the macrophage. These mice also provided a source of parasites that was ten fold greater than the standard BALB/c mice, greatly facilitating the isolation of surface molecules involved in entry.

Since I used *scid* mice as a source of parasites, the course of infection with *L. major* in these animals needed to be characterized. I demonstrated that the initial delay in infection, in *scid* mice compared with BALB/c mice, was not due to regulation by IFNγ, but is likely due to impaired inflammatory processes at the site of infection. In the later stages of infection, when more macrophages become available, the *scid* mice were exquisitely susceptible to the infection.

It is interesting to speculate about the mechanisms of disease progression in the *scid* mouse. Especially in light of the fact that the predominating theory for control of *L. major* infection is that individuals who resolve infection have a Th1 profile and non-healers a Th2 profile (Mossman *et al.*, 1986; Scott, 1989; Locksley and Scott, 1991). These two profiles are distinguished on the basis of the cytokines produced by their respective T-

cells. The *scid* mice have no functional B and T cells, yet despite the delay in infection these animals become heavily parasitized, suggesting that other mechanisms besides T cell cytokines are involved. It is known that in the resistant C57BL/6 mouse, the lesion site, early in infection with *Leishmania*, contains more mature macrophages and fewer immature macrophages, whereas mainly immature macrophages are present in the susceptible BALB/c mouse (Sunderkötter *et al.*, 1993; Mirkovich *et al.*, 1986). During the first two weeks of infection with *L. major* in athymic nude mice, there are more immature macrophages than mature macrophages, demonstrating that this phenomenon is T-cell independent (Sunderkötter *et al.*, 1993). Since immature macrophages are less activatable for intracellular killing they are a better cell for the survival of the parasite (Hoover and Nacy, 1984). It is likely that the BALB/c congenic *scid* mice also contain higher numbers of immature macrophages at the lesion site, thus propagating the growth of the parasite. Despite the absence of T cell produced lymphokines at the lesion site, other cells such as NK cells and mast cells may produce cytokines that are involved in propagating the infection in these immunodeficient animals.

As can be seen from the results of this study the promastigote and amastigote stages differed in receptors used to enter the macrophages. This was apparent in the susceptibility of the different macrophage populations to the parasite in vitro, and from the studies of blocking receptors for entry. Promastigotes from tissue culture were free of host proteins, whereas the amastigotes obtained from infected animals had host components bound to their surface. The importance of host components was evident by the observation that BALB/c and *scid*-source amastigotes were phagocytosed differently by the different macrophage populations, in vitro. This was due to the absence of IgG on the surface of *scid*-source amastigotes and the presence of IgG and greater amounts of host C3 on the surface of BALB/c-source amastigotes. The promastigotes obtained from in vitro culture had no host components bound to their surface for opsonized phagocytosis of the parasite. This is the first study to compare two sources of amastigotes; the normal BALB/c-source and the relatively 'clean' *scid*-source parasites.

Fresh serum was required to block entry of promastigotes of *L. major* using anti-CR3 mAbs. This demonstrates the importance of opsonization in the entry into macrophages through the CR3, by this stage of the parasite. This has been documented previously for *L. major* (Da Silva *et al.*, 1989; Mosser *et*

al., 1992). Although in certain species of *Leishmania*, such as *L. donovani*, entry of promastigotes through the CR3 occurs in the absence of serum (Blackwell *et al.*, 1985).

The parasite surface composition of promastigotes and amastigotes also differed. Few studies have been performed to examine the surface structure of amastigotes. Radiolabeling of *scid* amastigotes revealed three bands, of 13.8, 11.5 kDa, and a large band ranging from 4-9 kDa. Biotinylation of *scid*-source amastigotes revealed several additional bands not seen using iodination, a major band of 66, and minor bands of 57, 51, 32, 20 and 16 kDa. The major proteins that are exposed on the surface of promastigotes, 90, 80, 63, 50 and 25 kDa molecules (Jaffe *et al.*, 1984; Sacks *et al.*, 1985; Jaffe and Sarfstein, 1987; Hernandéz *et al.*, 1989), were not evident in amastigotes.

The molecules that predominate on the promastigote stage, LPG and gp63 are found at much lower levels in amastigotes and there is still debate as to whether gp63 is expressed (Medina-Acosta *et al.*, 1989; Frommel *et al.*, 1990; Pimenta *et al.*, 1991; Schneider *et al.*, 1992). Indirect evidence for surface exposed gp63, or a molecule with similar antigenic properties, in amastigotes was seen in this study. First, rabbit anti-promastigote gp63 reacted with *scid*-source amastigotes in an immunoblot. In fact, a stronger reaction was obtained in the immunoblot when the antigen source was detergent solubilized membrane proteins compared with the response to the whole amastigote. Second, the anti-gp63 antibody increased the uptake of *scid*-source amastigotes opsonized with the antibody, into macrophages in vitro. However, the gp63 antiserum detected two strong bands with equal intensity, of 59 and 84 kDa, in the *scid*-source amastigotes. The molecules may have shared epitopes with gp63 or may be a modified form of promastigote gp63, structurally different to suit the different environment encountered by the amastigotes. It is known that the promastigote and amastigote forms of LPG differ structurally; the phosphoglycan portion of LPG obtained from amastigotes of *L. major*, is larger than in promastigotes, and is not as anionic as the promastigote phosphoglycan portion of LPG (Turco and Sacks, 1991; Moody *et al.*, 1993).

The other major surface molecule of promastigotes, LPG, does not form a thick coat on the amastigote stage as it does in the metacyclic or infective promastigotes, in fact there is no thick coat evident on amastigotes (Pimenta *et al.*, 1991). There are fewer molecules of LPG on the amastigote surface, 6 x

10^4 molecules (Moody *et al.*, 1993), compared with the promastigote, 5×10^6 molecules (McConville and Bacic, 1989). Although LPG has been implicated in the binding of amastigotes to macrophages (Kelleher *et al.*, 1995) it is not known whether this molecule is used for phagocytosis.

In this study, differences were observed in the antigenic makeup of the different parasite stages and sources. Not surprisingly, the most striking differences were that the rabbit anti-promastigote antiserum recognized more bands with promastigote antigens than with amastigote antigen, conversely the rabbit anti-amastigote antiserum recognized more bands on amastigotes than promastigotes. It was also evident that there are antigens common to both parasite stages, because the sera from *L. major* infected mice detected numerous bands when promastigotes were used as the antigen, despite the fact that these mice were infected with amastigotes and had never been exposed to promastigotes.

A few comparative studies using either immune sera or mAbs have demonstrated differences and similarities in the antigenic structure of the promastigotes and amastigotes. (Handman *et al.*, 1984; Sadick and Raff, 1985; Handman and Hocking, 1982). This study also made a comparison of two sources of amastigotes that come from different hosts: the BALB/c mouse is immunocompetent, whereas the *scid* mouse is severely immunodeficient. Despite these differences in the hosts the antigenic structure of the two amastigote sources differed only slightly. Two bands of 146 and 134 kDa were observed when anti-amastigote antiserum was reacted with BALB/c -source amastigotes, but were not observed when the anti-serum was reacted with the *scid*-source amastigotes. It is unlikely that the 143 kDa band was host-derived because this band was also detected by sera from *L. major* infected mice; however, this possibility cannot be excluded. Another explanation for why scid-source amastigotes lack theses antigens is that the amastigotes, living in an immunodeficient host are not pressured by the host cell to express these antigens.

Studying the phagocytosis of the promastigotes and the two sources of amastigotes, it was evident that there was heterogeneity in the ability of the macrophage populations to phagocytize the parasites. Differences were observed in the phagocytosis of the promastigotes and amastigotes by a single macrophage population. This was not surprising, considering the differences in surface composition of the two stages of the parasite. However, differences

were also observed in the ability of different macrophage populations to phagocytize a single parasite stage or source. These differences in phagocytic ability of the macrophage populations reflect the heterogeneity of these cells, and were most likely due to differential expression of phagocytic receptors, since other factors such as genetic background of the cells or prior exposure with *L. major*, of the mice used to obtain the cells, did not change the ability of the macrophages to phagocytize the parasite.

Interestingly, a significantly lower percent of infection of promastigotes and *scid*-source amastigotes occurred in BMDM, yet opsonization significantly enhanced the entry of these parasites to levels equivalent to that of phagocytosis of BALB/c-source amastigotes. Peritoneal cells, on the other hand, phagocytized *scid*-source and BALB/c-source amastigotes equally, whereas very few promastigotes were phagocytized by these cells. This suggests that the FcR or the CR3 are extremely important in entry into BMDM, whereas different receptors are involved in entry into the peritoneal macrophages. Alternatively, peritoneal cells express higher levels of the receptors used by *scid*-source amastigotes in the absence of opsonization with IgG than do BMDM. BMDM are rarely used as the macrophage source in studying receptor mediated phagocytosis of *Leishmania*, and this was the first study to compare entry of the promastigotes and amastigotes into this macrophage population. A few studies examining peritoneal cells have demonstrated that the amastigote stage *L. major* is more readily phagocytized and survives better in young inflammatory macrophages (Fortier *et al.*, 1982; Pappas, 1983). It appears that the BMDM are also a good macrophage for entry and survival of amastigotes.

Both the FcR and CR3 were involved in the uptake of the amastigote stage into macrophages, whereas the MFR was not used by either amastigotes or promastigotes. Interestingly, blocking the FcRI and FcRII did not alter the uptake of amastigotes into macrophages, whereas simultaneous blocking of the FcRII and the CR3 increased the blocking above that observed with blocking the CR3 alone. These findings suggest that several receptors are involved in the entry and the amastigotes are flexible as to which receptor they use to enter the host cell.

It was also evident from this study, that entry through the FcR during a natural infection with *L. major* could involve the binding of antibodies specific to a number of different epitopes. The six anti-*Leishmania* anti-sera,

used in this study, contained antibodies that recognized different antigens, yet all enhanced the entry of amastigotes into macrophages. Studies of this nature have not been performed previously using amastigotes because of the lack of Ig-free parasites.

Although the MFR is not used by either stage of *L. major*, some form of carbohydrate-lectin interaction occurred at the interface of the parasite and macrophage. This was indicated by the reduction in the phagocytosis of promastigotes and *scid*-source amastigotes in the presence of carbohydrates. This effect was abrogated when *scid*-source amastigotes were opsonized with either IgG or GPS. Presumably, the IgG and complement components in the GPS masked the exposed carbohydrates, preventing their binding to carbohydrate receptors on the macrophage. It is well established that there are many surface exposed carbohydrates on both stages of the parasite (Gardiner and Dwyer, 1983; Wilson and Pearson, 1984). Therefore, it is very likely that other, as yet unidentified receptors may be involved in the entry of the amastigotes into macrophages.

I demonstrated that the amastigote stage of *L. major* uses the CR3 for entry, as do the promastigotes. The FcR is used by the amastigotes; however, the FcR would not be used by promastigotes in a natural infection. Although opsonization of promastigotes with parasite-specific antibody enhanced the phagocytosis of the parasite this receptor would not be used for promastigote entry during a primary infection with *Leishmania*, because there is no parasite-specific antibody at the site where the promastigotes are injected by the sandfly. On the other hand, during a chronic infection the lesion site contains B cells that can produce parasite-specific antibodies.

The levels of blocking seen in my study were never one hundred percent. A complication in performing receptor blocking studies is that many receptors have several ligand binding sites. For example, the CR3 contains binding sites for C3bi and LPS (Wright *et al.*, 1989; *Ross et al.*, 1985). Another difficulty arises from the fact that there is cooperation of receptors on the macrophage surface. Neutrophils from patients with leukocyte adhesion deficiency (LAD) that lack the β-chain of the CR3 fail to phagocytize IgG particles despite normal levels of FcR's (Gresham *et al.*, 1991). The CR3 and FcRII co-cap on neutrophils (Zhou *et al.*, 1993). The binding of modified α_2-macroglobulin to the MFR on macrophages enhances FcR mediated phagocytosis of IgG-opsonized red blood cells (Murai *et al.*, 1995).

Furthermore, not all receptor blocking reagents are effective in all macrophage populations. For example, the mAb M1/70 against the CR3, reduced the entry of amastigotes into resident peritoneal cells and J774A.1 cells but not into BMDM or inflammatory peritoneal macrophages. In addition, OAG enhances phagocytosis of C3b and IgG-coated particles by human monocyte-derived macrophages (Newman *et al.*, 1991), whereas in this study it did not alter the phagocytosis of either amastigote source into BMDM. Thus, the levels of blocking seen in my study may be partially due to receptor interactions and the inability to block all receptor binding sites.

Reviewing the literature, it is evident that there is a broad range in the percent of blocking of parasite entry into macrophages, reported in different studies. This is most likely due to the different macrophage populations, parasite and reagents that are used. It appears that higher levels of blocking are obtained when studying the promastigote stage compared with amastigotes. A 97% inhibition in promastigote entry occurred in macrophages adhered to mannan-coated slides (Wilson and Pearson, 1988). Mosser *et al.* (1987) reported a 90% inhibition of promastigote entry when the AGE-R and CR3 were blocked simultaneously. Adhering macrophages to mannan-coated slides resulted in an 80% reduction in promastigote entry, whereas a 60% reduction in entry was observed for amastigotes (Blackwell *et al.*, 1985). Greater levels of blocking using the promastigote stage may be due to simplified mechanisms of entry of this stage of parasite. The absence of host proteins on promastigotes, obtained from in vitro culture, may result in fewer choices for the parasite of macrophage receptors and receptor binding sites that can be engaged.

Blank *et al.* (1993) demonstrated an 80% reduction in the phagocytosis of amastigotes of *L. major* into Langerhan's cells, using anti-CR3 mAbs. However, these cells are not very phagocytic; only 20% of the cells became infected, and they are not suitable host cells since the amastigotes do not survive in them. Instead, it appears that the Langerhan cells are important in antigen processing for initiating immune responses. Hence, it would not be advantageous for the amastigotes to enter these cells, therefore amastigotes have probably developed strategies to enter the inflammatory macrophages that are elicited to the lesion site and that are suitable for survival of the parasite (Mirkovich *et al.*, 1986; Hoover and Nacy, 1984). A different combination of receptor ligand interactions are likely involved to ensure

entry into a 'safe haven'. *Leishmania* are very specific in inhabiting only macrophages; moreover, the different species of *Leishmania* show preferences towards different macrophage populations suggesting that there is a combination of specific receptors that are important in the phagocytosis of these parasites.

In a comparative study, by Blackwell *et al.* (1985), of promastigotes and amastigotes of *L. donovani*, blocking levels were reported that ranged from 15-40% for amastigotes in the presence of anti-CR3 mAb M1/70, whereas promastigote entry was blocked by 55-88%. These authors concluded that the low levels of blocking indicated that the CR3 receptor was not involved in phagocytosis of amastigotes (Blackwell *et al.*, 1985). I disagree with this interpretation, and believe that lower levels of blocking is further evidence that the amastigotes use the CR3, but can use a different receptor when it is blocked. The amastigote surface is more complex than the promastigote from in vitro culture, because the parasite is obtained from in vivo, and not only are there different parasite molecules exposed to the macrophage receptors but host proteins are present allowing alternative interactions with macrophage receptors. The lower percent blocking observed in their study and mine suggests that the amastigotes can bind to different receptors and are not reliant on a single receptor for entry. For example, *scid* mice lack IgG, therefore FcR-mediated phagocytosis does not occur in these animals, yet they are highly susceptible to infection with *L. major*.

The major surface exposed proteins on amastigotes in this study were molecules of very low molecular weight. The molecule that blocked entry of amastigotes into macrophages was also a low molecular weight protein. To my knowledge, this protein has not been isolated previously from either stage of the parasite. An 11 kDa surface molecule has recently been identified in all species of *Leishmania* and many members of the trypanosome family (Tolson *et al.*, 1995; Jardim *et al.*, 1995). However, mAbs to this protein did not detect the blocking molecule from this study. The blocking of entry by the parasite molecule in this study was most likely due to the parasite ligand binding to macrophage receptors, resulting in blocking the binding of the live amastigotes. The level of blocking, 30%, may possibly be increased with the addition of more of the parasite ligand; however I do not expect to obtain absolute blocking, as this feature was never observed during my study. It is likely that the parasite ligand is only one of several potential parasite ligands

involved in binding to macrophage receptors, since, as was shown, several receptors are used in phagocytosis of the amastigote. This suggests that more than one parasite ligand is important for phagocytosis to occur.

A single study examined blocking entry of *L. major* amastigotes by an amastigote molecule, the glycolipid LPG (Kelleher *et al.*, 1995). They obtained high levels, 80%, of reduction in binding of amastigotes to macrophages. They did not report whether phagocytosis of the amastigotes was also reduced. However, it remains to be determined whether the blocking was entirely due to the occupation of the receptor site thereby preventing binding of the live parasite, or due to triggering intracellular events that altered the phagocytosis process. Purified LPG has strong effects on macrophages, for example, it impairs signal transduction through the protein kinase C pathway (McNeely and Turco, 1987; Descoteaux *et al.*, 1991; 1992).

Although the blocking molecule described in the present study was obtained from the water soluble fraction of amastigotes, there was indirect evidence suggesting that the blocking molecule originated from the membrane. 1) Low molecular weight proteins were the major surface components that were labeled with ^{125}I . 2) A 13 kDa molecule was observed in the supernatant containing the water soluble fraction of ^{125}I-labeled amastigotes. 3) Low molecular weight molecules were a major component of the detergent solubilized membrane of amastigotes. 4) The blocking molecule was strongly recognized by a rabbit anti-serum against the integral membrane of *scid*-source amastigotes. Further studies are needed to determine the purity of the blocking molecule contained within the FPLC fraction that was used to block entry and to characterize the chemical nature of the protein. However, the information I have so far indicates that this may be a novel molecule.

I undertook a reductionist approach to try and isolate some of the interactions that occur at the interface of the membranes of the amastigote and its host cell. However, the interactions in the mammalian host are more complex, involving many different variables. The inflammatory lesion site contains not only macrophages but several types of immune cells: T and B lymphocytes, natural killer cells, eosinophils (Heinzel *et al.*, 1988; Esterre *et al.*, 1992; McElrath *et al.*, 1987) and non-immune cells. The multitude of different cytokines at the lesion site can affect the expression and behavior of phagocytic receptors. Moreover, extracellular matrix proteins can act as

opsonins for the parasite as well as modify the function of phagocytic receptors.

This study has provided new insight into the receptor interactions with the amastigote stage of *L. major*, by identifying several receptors involved in the entry of amastigotes, as well as identifying a molecule involved in the entry of the parasite into macrophages.

FUTURE DIRECTIONS

Research into understanding the biology of amastigotes and the mechanisms whereby they cause disease in their host is still in its infancy. If I were to continue research on this topic I would concentrate on isolating and characterizing the blocking molecule described in this study. There are several research areas that could be explored using this molecule as a probe. For example, it could be studied as a new vaccine candidate, or used to study the survival of amastigotes within the macrophage and the modulation of the host's defenses. However, I would concentrate on the use of this molecule to further characterize the interaction at the cell surface for receptor-mediated entry of *Leishmania* into the macrophage.

The first step would be to determine whether the molecule is of surface origin, and if so, the ligand would be purified from the amastigote membrane. Once purified in sufficient amounts, the physicochemical characteristics could be determined. Antibodies made against this protein would be of use in identifying the expression levels throughout the life cycle of the parasite, and for determining species and genus specificity of the molecule. The amino acid sequence and antibodies could be used as a probe for screening a cDNA library to obtain the gene sequence and eventually produce recombinant protein to obtain large amounts of material.

To further understand the role of the parasite ligand in the biology of amastigotes, the ligand should be studied to determine whether it can be opsonized with various host components such as C3, IgG, or extracellular matrix proteins such as fibronectin or laminin, in addition to binding directly to the macrophage receptor as was suggested by this study. Evidence from this study suggests that the ligand is not antigenic in mice, because the sera from mice infected with *L. major* did not recognize the low molecular weight

antigens in immunoblots, unlike the rabbit antisera which detected several of the low molecular weight proteins.

Once sufficient quantities of purified parasite ligand is available, the kinetics of the receptor ligand interactions can be studied in vitro, to determine the number of receptors involved in binding and the affinity of the ligand for the receptor(s). The purified molecule can be used to identify the receptor by cross-linking to macrophage receptors using the photoaffinity labeling technique (Clark and Armstrong, 1990). To date, this approach has not been used in studying macrophage receptors used by *Leishmania.* Upon identification of the receptor, further verification of the role of the receptor in phagocytosis of amastigotes can be done by introducing its gene into a non-phagocytic cell that lacks the specific receptor. These transfected cells can be used to study the blocking of the uptake of amastigotes by the parasite ligand. This method has been successfully used in studying the uptake of *Pneumocystis carinii* by the MFR introduced into COS cells (Ezekowitz *et al.*, 1991), and the binding of promastigotes to the CR3 (Mosser *et al.*, 1992). Having identified the receptor(s) one could then pursue studies to determine the effects of binding of the ligand on signaling events triggered by the receptor.

Research into the effects of cytokines on the receptor-ligand interaction would be of value in understanding the mechanisms of disease occurring at the lesion site. It is well established that cytokines modify receptor expression. IFNγ decreases expression of the MFR (Moekena and Gordon, 1985; Ezekowitz *et al.*, 1986), whereas it increases FcR expression on macrophages (Guyre *et al.*, 1983; Perussia *et al.*, 1983). IFNγ increases binding of Ig coated red blood cells (Wright *et al.*, 1986), but, the phagocytosis of Ig opsonized red blood cells is decreased (Wright *et al.*, 1986; Jungi *et al.*, 1987). This is explained by the fact that the expression of high affinity Fc receptors is enhanced, but the expression of the low affinity receptors used for phagocytosis remain unaltered. The expression of CR1 and CR3 on human monocytes is not altered by IFNγ; however, the ability of these receptors to bind C3b and C3bi coated red blood cells is significantly reduced (Wright *et al.*, 1986). Hence, cytokines can modulate the receptors FcR and CR3 used for entry of amastigotes of *L. major.*

In vitro studies have revealed that macrophages can be made resistant to infection by stimulation with appropriate cytokines. IFNγ in combination

with other cytokines induces macrophages to resist infection with amastigotes of *L. major* (Davis *et al.*, 1988). The other cytokines that cooperate for resistance to infection are IL-2, IL-4 and GM-CSF (Belosevic *et al.*, 1988; Belosevic *et al.*, 1990). Studying the mechanisms involved in cytokine-induced resistance to infection, whether it occurs through altered levels of receptor expression or modulation of triggering events following binding of the parasite, would provide a means of modulating established infections.

Another area for study that would ensue from the identification of specific receptors used by *L. major* is the role of extracellular matrix proteins in phagocytosis of *L. major*. It is known that fibronectin enhances the uptake of both promastigotes and amastigotes of *L. major* into macrophages (Wyler *et al.*, 1985). Extracellular matrix proteins can enhance phagocytic activity of macrophages by mechanisms distinct from the phagocytic receptors. For example, the adhesion of macrophages to fibronectin or laminin coated surfaces enhances the uptake of particles by the CR3 and the FcR (Pommier *et al.*, 1983; Bohnsack *et al.*, 1985). As mentioned above, IFNγ reduces the ability of CR1 and CR3 on macrophages to bind complement components; this effect is abrogated when the macrophages are adhered to fibronectin coated slides (Wright *et al.*, 1986). Therefore, extracellular matrix proteins can modify the phagocytosis of organisms; however, the mechanisms by which this occurs are not fully understood (for review see Brown, 1995). Having shown that the FcR and CR3 are involved in amastigote entry, the effect of extracellular matrix proteins on these receptors could be studied in the presence of the live parasite and purified parasite ligand.

The blocking of receptors could also be studied in vivo, to determine whether blocking entry of amastigotes reduces the severity of the disease. Over-expression or under-expression of the gene in transfected organisms would provide an alternative technique to study the mechanisms of entry and the involvement of a specific molecule. Several transfection systems are available for use with *Leishmania* (Laban *et al.*, 1990; Cruz and Beverley, 1990).

Several other interesting approaches have evolved from the knowledge of parasite ligands and their host cell receptors in targeting cells for chemo-and gene therapy. For example, coupling of antileishmanial drugs to a ligand for the scavenger receptor or the MFR on macrophages significantly reduces infection with *L. donovani* in vitro and in vivo

(Mukhopadhyaya *et al.*, 1989; Nègre *et al.*, 1992). An alternative approach is the use of the malarial circumsporozoite protein as a carrier to introduce DNA into hepatocytes (Ding *et al.*, 1995).

Thus, identification of a novel amastigote ligand involved in binding macrophage receptors would provide an important probe for studying receptor-mediated phagocytosis, the event that initiates infection and results in disease with this intracellular pathogen.

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APPENDIX I: Immunization schedules to obtain immune sera.

Immunization Schedule to obtain serum against BALB/c amastigotes of the strains NIH 80 and NIH 173:

Rabbits were injected in the subscapular and intramuscular regions with whole amastigotes from BALB/c mice. Each immunization dose contained 5 x 10^8 dead cells which were killed by repeated freeze thawing.

Week 0 5x10^8 amastigotes in complete Freund's adjuvant (CFA)(Gibco)
Week 3 " " in incomplete Freund's adjuvant (IFA)(Gibco)
Week 7 " " in IFA
Week 11 " " in IFA
Week 13 serum was collected.

Immunization Schedule to obtain serum against *scid* amastigotes of the strain NIH 173:

Rabbits were injected in the subscapular and intramuscular regions with whole amastigotes from *scid* mice. Each immunization dose contained 5 x 10^8 dead cells which were killed by repeated freeze thawing.

Week 0 5x10^8 amastigotes in CFA
Week 2 " " in IFA
Week 5 " " in IFA
Week 7 serum was collected.

Immunization schedule used to obtain sera against PNA$^-$ promastigotes and *scid* amastigote integral membrane proteins:

To obtain anti-promastigote serum, each immunization dose contained 5 x 10^8 dead cells which were killed by repeated freeze thawing. Triton X-114 solubilization was used to obtain integral membrane proteins and each dose consisted of 100 µg protein.

Week 0 antigen in CFA

Week 3 " in IFA

Week 5 " in IFA

Week 8 serum was collected.

APPENDIX II: The mouse strain and method used to produce macrophage-like cell lines.

LINE	DIFFERENTIATION STATE	MOUSE STRAIN	METHOD OBTAINED
J774A.1	Mature Macrophage	BALB/c	Tumor
P388D.1	"	DBA/2	Methylcholanthrene induced tumor
ANA-1	"	C57BL/6	J2 retrovirus infected bone marrow
BALBMQ clone BA-1	"	BALB/c	" "
M1	Myeloblast	SL	Spontaneous myeloid leukemia
WEHI 3	Immature Macrophage	BALB/c	Mineral oil induced tumor